University of Alberta

# Protein Mass Mapping for Bacterial Identification

by

Lidan Tao ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Chemistry

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Protein Mass Mapping for Bacterial Identification** submitted by Lidan Tao in partial fulfillment of the requirements for the degree of Master of Science.

Dr. Liang Li (Supervisor)

Dr. John-Bruce Green

Dr. Peter Sporns

Date: Sept. 26, 2003

# Abstract

A protein mass mapping approach using mass spectrometry (MS) combined with an experimentally derived protein mass database is presented for the rapid and effective identification of bacterial species. A prototype mass database from the protein extracts of nine bacterial species has been created by off-line high performance liquid chromatography (HPLC) matrix-assisted laser desorption ionization (MALDI) MS, in which the microbiological parameter of bacterial growth time is considered. The applicability and reliability of this protein mass mapping approach has been tested with seven unknown bacterial species in a single blind study, and identification results with 100% accuracy were obtained. Our studies have demonstrated that the major advantages of protein mass mapping approach include its relatively fast data acquiring and analyzing speed, apparent simplicity of the experiments involved, not dependence on the availability of genome or proteome sequence information, and remarkable sensitivity.

*To my parents and my husband, with all my love*

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## List of Abbreviations

| | |
|---|---|
| HCCA | α-cyano-4-hydroxycinnamic acid |
| TFA | trifluoroacetic acid |
| ACN | acetonitrile |
| ATCC | American type culture collection |
| *E.coli* | *Escherichia coli* |
| LB | luria broth |
| 2D PAGE | two-dimensional polyacrylamide gel electrophoresis |
| HPLC | high performance liquid chromatograph |
| MALDI | matrix-assisted laser desorption/ionization |
| ESI | electrospray ionization |
| MS | mass spectrometry |
| Py-MS | pyrolysis mass spectrometry |
| FAB | fast atom bombardment |
| TOF | Time-of-Flight |
| Q | quadrupole |
| IT | quadrupole ion trap |
| TIC | total ion chromatogram |
| UV | ultraviolet |
| fwhm | full width at half maximum |
| LPS | lipopolysaccharides |
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |

| | |
|---|---|
| rRNA | ribosomal ribonucleic acid |
| MW | molecular weight |
| m/z | mass-to-charge-ratio |
| nL | nanoliter (1 μL = $10^{-9}$ L) |
| μL | microliter (1 μL = $10^{-6}$ L) |
| mL | milliliter (1 mL = $10^{-3}$ L) |
| mg | milligram (1 mg = $10^{-3}$ g) |
| Å | angstrom (1 Å = $10^{-10}$ m) |
| μm | micrometer (1 μm = $10^{-3}$ m) |
| mm | millimeter (1 mm = $10^{-3}$ m) |
| g | force |
| pg | picogramme (1 pg = $10^{-12}$ g) |
| ppm | part per million |
| kDa | kilodalton (1 kDa = $10^{3}$ Da) |
| kV | kilovoltage (1 kV = $10^{3}$ V) |

# Chapter 1

# Introduction

## 1.1 Bacterial Identification

There is currently an increasing need to establish more efficient and accurate bacterial identification methods due to their significant importance in the areas of microbiology, pharmaceutical research and drug development, clinical treatment and disease diagnoses, agriculture, environmental and food science, as well as homeland security and biological warfare detection. As the simplest and oldest life form, bacteria exist wherever there is life on Earth: in soil, water, and air, and even extreme conditions such as in arctic ice and volcanic vents! Bacteria fall into a category of life called the *Prokaryotes*, small cells with relatively simple internal structures. *Eukaryotes* are larger and often multi-celled organisms that contain a complex array of subcellular organelles including nuclei.

Species demarcation in bacteria has exclusively relied on the *phenotypic* and *genotypic* clustering of bacterial strains. Bacterial species have long been understood to be phenotypic clusters based largely on metabolic characters [1-5]. Numerical taxonomy has clearly demonstrated that bacterial strains fall into discrete phenotypic clusters, providing a means for discovering species and for classifying unknown strains into already characterized species [6]. Since the emergence of molecular biology in the 1930's, genotypic clustering has gradually replaced phenotypic clustering as a primary criterion for bacterial species demarcation. For decades, clustering based on whole-genome DNA-DNA hybridization between pairs of strains has served to distinguish species [5, 7-9]. More recently, systematists are using clustering of 16-rRNA and protein-coding gene sequence as criteria for species demarcation [5, 10, 11]. Techniques in demarcating bacterial species are well-established, and therefore in general, are deemed as a task of determining the relevant phenotypic/genotypic characters of unknown bacteria and matching them with an appropriate database containing information about known taxa. Prior to any further discussion on this respect, it is necessary to introduce some relevant background information on the biological features of bacteria.

### 1.1.1 Important Biological Features of Bacteria

Bacteria are unicellular organisms that appear in many shapes and sizes, from minute spheres, cylinders and spiral threads, to flagellated rods, and filamentous chains. A bacteria cell generally has the following major structures: capsule, cell wall, cytoplasmic membrane, cytoplasm, ribosomes, nucleoid, pili, and flagella (as shown in Figure 1.1).

In some species of bacteria, a third protective covering capsule is also observed, which is made up of polysaccharides. The most important role of capsules is to keep the bacterium from drying out and to protect it from phagocytosis by larger microorganisms. Moreover the capsule is a major virulence factor in disease-causing bacteria such as *Escherichia coli* and *Streptococcus pneumoniae*. Each bacterium is enclosed by a rigid cell wall composed of peptidoglycan, a protein-sugar (polysaccharide) molecule. The wall gives the cell its shape and surrounds the cytoplasmic membrane, protecting it from the environment. The strength of the wall is responsible for keeping the cell from bursting when there are large differences in osmotic pressure between the cytoplasm and

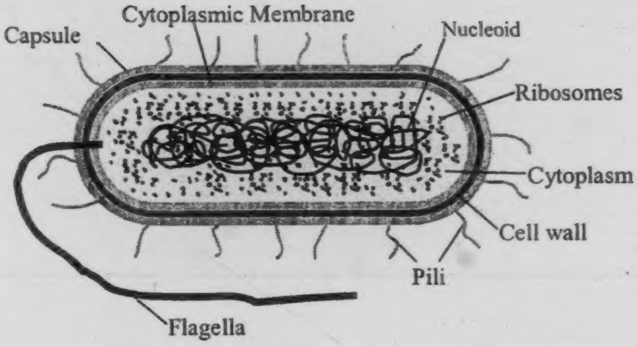

**Figure 1.1** An illustration of bacterial cell structure.

the environment. Cytoplasmic membrane is a layer of phospholipids and proteins. It is a critical permeability barrier, separating the inside of the cell from the outside and regulating the flow of materials. The functions for cell growth, metabolism, and replication are carried out in the cytoplasm (or protoplasm), a gel-like matrix composed of water, enzymes, nutrients, wastes, and gases and containing cell structures such as ribosomes, a chromosome, and plasmids (smaller circular auxiliary DNA strands). The nucleoid is a region of cytoplasm where the chromosomal DNA is located, usually in the form of a single, circular chromosome. It is not a membrane bound nucleus, but simply an area of the cytoplasm where the strands of DNA are found. Ribosomes are small particles comprised of protein and RNA. They are part of the translation apparatus responsible for the synthesis of cellular proteins. Bacterial ribosomes are never bound to other organelles as they sometimes are in eukaryotes, but are freestanding structures distributed throughout the cytoplasm. Many bacteria have flagella, hair-like structures that provide a means of locomotion. They can be found at either or both ends of a bacterium or all over the surface. Many species of bacteria have pili, small hair-like projections emerging from the outside cell surface. These outgrowths assist the bacteria in attaching to other cells and surfaces, such as teeth, intestines, and rocks. Without pili, many disease-causing bacteria lose their ability to infect because they are unable to attach to host tissue.

Bacteria are usually semitransparent and are so small that they need to be viewed under a microscope using special stains. The traditionally used stain is called the "Gram stain." In this method, methyl violet stain is poured over bacteria that have been spread out thinly on a microscope slide and heat-killed. The stain is washed off with a solvent and a counterstain is applied. Bacteria that maintain the original methyl violet stain are called *Gram positive*, whereas bacteria that lose it and take up the counter-stain are called *Gram negative*. The differential staining is due to differences in the physical structures of the cell wall.

Cell wall composition varies widely among bacteria and is one of the most important factors in bacterial species analysis and differentiation. A thick peptidoglycan layer constitutes most of the Gram-positive cell wall (see Figure 1.2A). In contrast, the cell walls of Gram-negative bacteria are more complex in structure than the Gram-positive forms (see Figure 1.2B). Outside of the cytoplasmic membrane (cell membrane) is a thin layer of peptidoglycan. External to the peptidoglycan is an additional membrane, the outer membrane. The outer membrane is a lipid bilayer similar to the cell membrane, containing lipids and proteins, as well as lipopolysaccharides (LPS). A list of the main differences between Gram-positive and Gram-negative cells is outlined in Table 1.1.

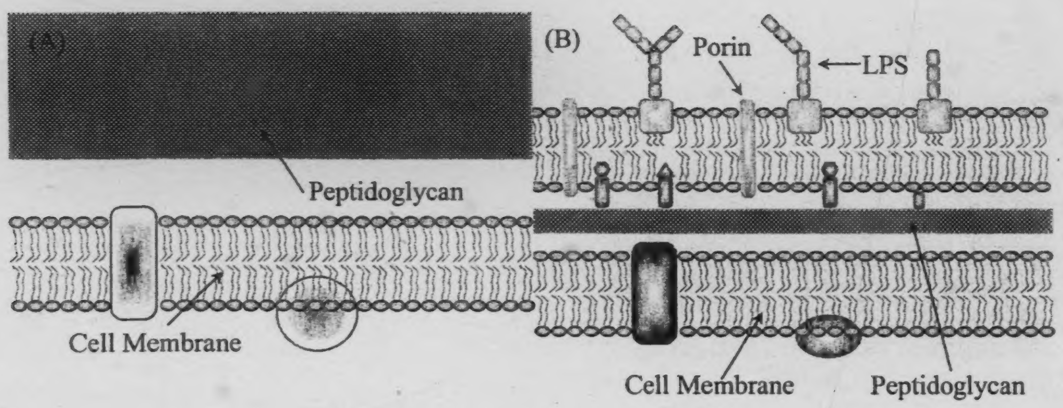

**Figure 1.2** Structure of cell wall. (A) The Gram-positive cell wall. (B) The Gram-negative cell wall.

**Table 1.1** Difference between Gram-positive and Gram-negative cells

| Property | Gram-positive cell | Gram-negative cell |
|---|---|---|
| Thinkness of wall | 20-80 nm | 10 nm |
| Number of layers in wall | 1 | 2 |
| Peptidoglycan content | >50% | 10-20% |
| Teichoic acid in wall | + | − |
| Lipid and lipoprotein content | 0-3% | 58% |
| Protein content | 0% | 9% |
| Lipopolysaccharide | 0% | 13% |

Bacteria reproduce themselves by a process called binary fission: each cell increases in size and divides into two cells. During this process there is an orderly increase in cellular structures and components, replication and segregation of the bacterial DNA, and formation of a septum or cross wall that divides the cell into two progeny cells. Bacterial requirements for growth include sources of energy, "organic" carbon (*e.g.*, sugars and fatty acids), and metal ions. Optimal temperature, pH, and the need or lack of need for oxygen are important as well. In the laboratory, bacteria are grown in culture media, which is designed to provide all the essential nutrients in solution for bacterial growth

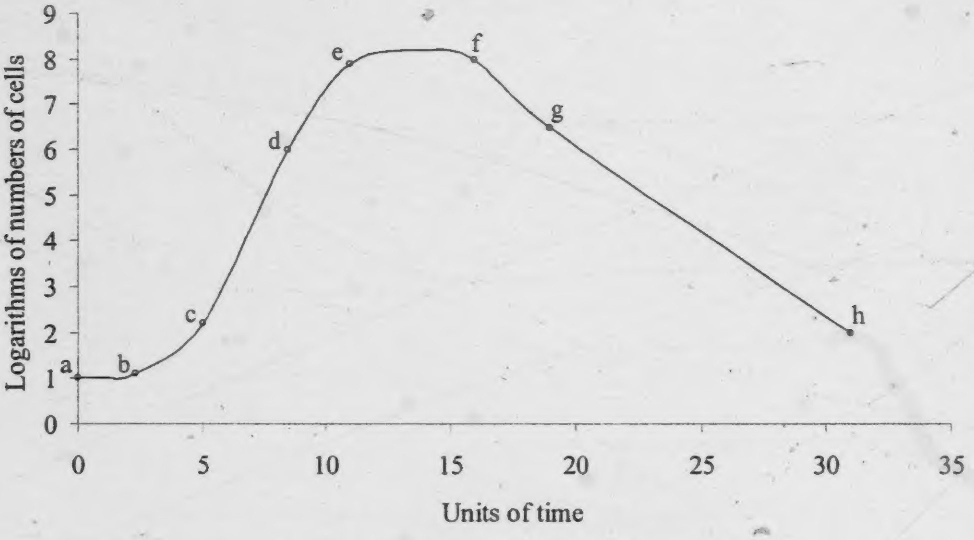

**Figure 1.3** A typical growth curve of bacterium.

under proper conditions. When bacteria are grown in a closed system (also called a batch culture), the population of cells, in most cases, exhibits a growth dynamics as illustrated in Figure1.3. This is a result of variations in the environment such as decreasing in pH of the culture due to formation of acid products, exhaustion of nutrient supply, and accumulation of waste. The first stage is initial stationary phase (ab) where cells adjust to the new medium. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, *etc.*, and increasing in metabolic activity. Subsequently, cells start dividing regularly by binary fission once they have reached a critical size. The rate of multiplication in this lag phase (bc) increases with growth time. The next stage is logarithmic growth phase (cd). In this phase, there is no change in the average cell mass, while the number of cells is increased and the rate of multiplication does not change. Next, the cells enter the phase of negative growth acceleration (de) where the rate of multiplication starts decreasing. The cells continue to increase in numbers at a lower rate than the logarithmic growth phase. After this, the rate of cell division equals the rate of cell death, or the population of cells has simply ceased growing and dividing, and therefore the number of living cells remains constant. This phase is called maximum stationary phase (ef), which usually occurs when the cell concentration is so great that some aspects of the environment are no longer able to serve the requirements of logarithmic growth. After a certain period, the number of living cells rapidly decreases, indicating the onset of accelerated death phase (fg). Finally, cell growth remains at the logarithmic death phase (gh) where the rate of death remains constant. The number of viable cells decreases exponentially, essentially the reverse of growth during the logarithmic phase. The cells assume a variety of unusual shapes, which makes them difficult to identify.

### 1.1.2 Bacterial Identification Methods

The range of characters employed for bacterial identification has been kept increasing. Nonetheless, there are mainly four categories for bacterial identification: 1) morphologies and behaviors such as gross and fine structure, motility, physiology, enzyme production, and nutrition, 2) cell components that include wall composition, lipids and whole organism pyrolysis, 3) proteins that include amino acid sequences, serology, *etc.*, and 4) nucleic acids, for example, DNA-DNA hybridization, DNA and rRNA sequencing, DNA fingerprinting, and nucleic acid probes. Based on the characters chosen, bacterial identification methods may be divided into three classes, namely computer-assisted identification, nucleic acid probes, and chemotaxonomy.

Computer-assisted or numerical identification are mainly applied to data derived from category 1 (above). In this method, a diverse set of morphological and biochemical characters from an unknown sample are referenced to a database which should comprise at least 100 characters and perhaps 20-40 taxa using computer software [9, 12-13]. A major innovation occurred with this method when manual miniaturized identification systems were introduced in the late 1960's. Among these, the API 20E strip test is one still commonly used in clinical microbiology labs throughout the world, and is considered to be the "gold standard" commercial system even though it is a manual method [14]. Recently, several automated systems such as AS-3/touchSCAN system and Vitek AutoMicrobic system have been developed [15]. Significantly, they reduce the time required for identification of rapidly growing bacteria from one or more days to 2-4 h. These commercial systems provide fast and accurate identification results for many common species in a cost-effective manner. However, there are some inherent drawbacks using these systems to identify bacterial species [16]. First, biochemical attributes often do not accurately reflect the genomic information of a given species. Moreover, phenotypic characters may be unstable with changes in environmental conditions, *e.g.*, growth medium, pH levels, and temperature. Second, commercial systems usually provide definitive identification only for well-studied groups of organisms. Biochemical tests included on the panel of commercial systems cannot be renewed with the discovery of newer species.

Recent advances in molecular biology and gene technology are creating many exciting opportunities for rapid and more reliable bacterial identification. For example, a nucleic acid probe is a fragment of single-stranded nucleic acid designed to hybridize with a highly specific, complementary bacterial sequence. The nucleic acid probe method provides a fast and specific bacterial identification at all levels and is especially useful for the identification of bacteria in complex biological specimens, for example, face, blood, and urine, or in environmental samples such as water and food [17]. Many different approaches are used to prepare nucleic acid probes. Early studies used whole-cell DNA [5, 7-9, 18-20], while later on, more specific probes of random DNA fragments were designed [21]. Unfortunately, this method uses DNA-DNA hybridization, an expensive, technically complex, and labor-intensive procedure. Recently, sequencing of the small subunit (16S) and (23S) rDNA has become commonplace in nucleic acid probe methodology [5, 10-11, 22-25]. At present, 16S rDNA is the most common molecular technique used for bacterial identification. Although the 16S rDNA gene sequence is a powerful tool for bacterial identification, it still faces several problems and challenges [16]. Since ribosomal genes are highly conserved, rDNA sequence variation between strains is less evident than with DNA pairing studies. In addition, there are no consensus guidelines that define what constitutes a species based on 16S rDNA gene sequence similarity or divergence. Strains with 97% or greater similarity may or may not belong to the same species.

Chemotaxonomy, or chemosystematics, is a rapidly developing area where information derived from categories 2 and 3 are used to identify bacteria. Most bacterial taxonomies can be categorized based on chemical properties [26]. For example, difference in the chemical composition of cell wall polymers results in various morphological features. Chemical markers can be used at all taxonomic levels, though some groups found that their discriminatory power may vary between taxa [27-28].

Serological identification represents the earliest of the chemotaxonomic methods. Since the antibody recognizes chemical structures within the antigen, the reaction of an antiserum with an antigen or antigens from microorganisms is used to classify bacteria. Serology is often the final stage of identification to produce conclusive evidence of the identity of an organism, and remains one of the few techniques that allow direct detection of specific organisms within a mixture in clinical and environmental samples. There are several drawbacks for serological identification. First, it is a technically complex method. Second, it is not always possible to quantify the interaction of antibodies with an organism. Third, cross-reactions still exist even when a monoclonal reagent, which is more specific than polyclonal antisera, is employed.

Chemotaxonomy has been developing with the introduction and application of reliable, rapid and sensitive analytical methods, such as chromatography, electrophoresis, and mass spectrometry (MS). Chemotaxonomy using MS, rather than other time-consuming and laborious methods has become a dark horse among many other methods due to its particular advantages of rapidity, high mass accuracy, high sensitivity, high resolution, simple sample preparation, and less sample consumption. So far, it has turned into a very promising method for bacterial identification in many applications. A detailed discussion on bacterial identification using MS is introduced in the next section.

## 1.2 An Overview of Bacterial Identification Using Mass Spectrometry

During the past decades, mass spectrometry (MS) technique has played an increasingly important role in bacterial identification largely because of its remarkable speed of data acquisition and less sample consumption. The identification of unknown bacteria using MS primarily relies on profiling the characteristic chemical biomarkers of a bacterium as molecular signatures. In 1975, Anhalt and Fenselau employed electron ionization to identify small molecules from heating dehydrated bacteria [29]. This approach was later developed to pyrolysis mass spectrometry (Py-MS), one of the whole-organism fingerprinting techniques for bacterial identification [30-32]. Pyrolysis is defined as the thermal breakdown of complex organic molecules in an inert atmosphere to produce low molecular weight fragment of the original material. In Py-MS, the resulting fragments are characteristic of bacteria and can be resolved to produce a fingerprint using MS for the identification of bacteria. However the pyrolysis data typically appear as a complex spectrum so that the observed ions are not directly reflecting the biological information of the corresponding bacterial cells [33].

With the development of fast atom bombardment ionization (FAB), polar membrane lipids were used as a useful biomarker for bacterial identification [34-37]. In this approach, the profiles of the lipid constituents in the bacteria were matched to a database of reference spectra. FAB-MS is superior to the Py-MS in several respects, particularly in its ability to generate high-molecular-weight ions. But the spectra are still complex.

More recently, the development of soft ionization methods, such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have advanced the capability of MS for bacteria identification by generating ions of larger molecules such as proteins [33, 38-59], DNA [60-68], and oligosaccharides [69] without significant decomposition or fragmentation. Among those larger molecular markers, proteins from both cell extracts [38-48] and whole cells [33, 49-59] have drawn particular attention not only due to their significant abundance in the bacterial cells (*ca.* 50% of the dried cell weight) but also because they can confer indirect genetic information about the specific bacterial species studied. Hence, MS analysis of proteins has become an important approach for unambiguous bacterial identification.

Initially, MS-based bacterial identification methods were based on the comparison of a mass spectrum obtained from an unknown species to those detected from known bacteria [38-40, 44, 49-52, 70]. A relatively simple procedure it is, but this comparison method often leads to uncertain or even erroneous results. To overcome this drawback, Jarman *et al.* adopted a fingerprint mass spectral library approach [71]. Even though the method is, to some extent, an improvement for the above approach, it is still devoid of accuracy and reproducibility mainly because the MS spectral appearance can vary significantly with experimental conditions [40, 53, 54, 57]. Recently, a "robust" approach of comparing protein masses in the spectrum of an unknown sample to an internet-accessible protein database has emerged, in which mass spectral reproducibility is no longer the key factor for correct bacterial identification [72]. Despite some modifications and enhancements, this approach is still facing several severe challenges [73-75]. Firstly, the number of bacteria with complete genome sequence information is relatively limited in public databases. Secondly, these bacteria span the range from incomplete to virtually complete protein mass entries. Use of these databases for matching purposes to an experimental mass spectrum has an inherent statistical bias in the identification and discrimination results. Finally, the protein masses are likely to be different from those derived from the genome due to complex biological processes such as post-translational modifications.

As an alternative, a novel and promising approach of protein mass matching with an experimentally derived mass spectral database has been devised, which gives significant advantages compared to the aforementioned MS-based methods [76]. The essence of this method can be summarized as matching a set of protein masses in a mass spectrum from an unknown species against an experimentally derived protein mass database of many known bacteria. The significance of using this method is that the experimentally derived mass database can be established rapidly and readily adopted, and it does not rely on the availability of genome or proteome sequence information. Since this database is generated from experiments, it is capable of reflecting a plethora of post-translational modification information that is essential for bacterial identification.

## 1.3 Some Useful Mass Spectrometric Techniques for Bacterial Identification

Mass spectrometry is the measurements of m/z of the ionized analytes in gas phase. A mass spectrometer normally consists of an ion source where analytes are converted to gas-phase ions, a mass analyzer that separates those ions according to their m/z ratio, and a detector that registers the number of ions at each m/z value.

Of many ionization techniques for mass spectrometry, Py-MS and FAB-MS were extensively used in the early bacterial MS profiling. However, these "hard" ionization methods were often insufficient to provide direct information on the constituents of the bacterial cells. Nowadays, they have been largely replaced by the new "soft" ionization methods, namely matrix-assistant laser desorption ionization (MALDI) and electrospray ionization (ESI), which allow the detection of much larger molecules, e.g., DNA, protein, peptides, and oligosaccharides. In my research, MALDI time-of-flight (TOF) and ESI quadrupole mass spectrometry have been extensivly used. Fundamentals of these two MS techniques are therefore described in the next section.

### 1.3.1 MALDI Time-of-Flight (TOF) MS

MALDI-TOF mass spectrometer is probably one of the most powerful tools in proteomics field. In 1987, two research groups published independently a new ionization method named MALDI [77-80]. During the past decade, MALDI has proven to be one of the most successful ionization methods for mass spectrometric analysis and investigation of large molecules. It is a method that allows for vaporization and ionization of non-volatile biological samples from a solid-state phase directly into the gas phase. The mechanism of MALDI is not completely understood, but it is widely accepted that the ionization of protein molecules occurs via gas phase proton-transfer reactions between excited matrix molecules and analyte molecules (see Figure 1.4) [81]. The sample (analyte) is usually first mixed with a proper matrix solution to achieve a molar ratio of matrix to analyte ≥ 500:1. Matrices are generally small organic

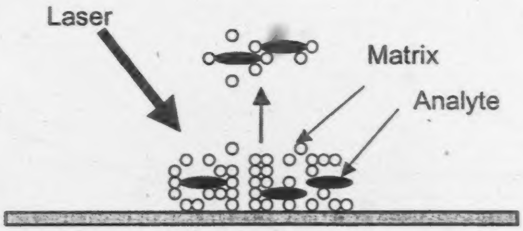

**Figure 1.4** Schematic illustration of the principle of MALDI.

compounds. The resulting mixture is then deposited onto a MALDI sample target. After solvent evaporation, the analyte and matrix form co-crystals on the target. The analyte molecules are distributed throughout the matrix such that they are completely isolated from one another. Normally, an ultraviolet (UV) laser beam (337 nm) serves as desorption and ionization source in MALDI. Upon irradiation, the matrix molecules efficiently absorb a large amount of energy, minimizing sample damage and ion fragmentation, causing rapid vibrational excitation, and forming clusters made up of a single analyte molecule surrounded by neutral and excited matrix molecules. Subsequently, the matrix molecules evaporate away from these clusters to leave the excited analyte molecule. The analyte molecules can become ionized by simple protonation by the photo-excited matrix, leading to the formation of the typical $[M+H]^+$. As the matrix molecules expand into the gas phase rapidly, the entrapped analyte molecules (or molecular ions) are also transported into the gas phase.

MALDI has some characteristics that are favorable for the analysis of biological samples. It has high tolerance to high concentration of impurities and dissolved salts, which results in simple sample preparation and fast analysis. Additionally, MALDI is very sensitive for proteins and peptides. Time-of-flight mass analyzers are the most common choice of analyzers coupled to MALDI ion sources. Figure 1.5 shows the basic principle of a linear TOF analyzer. The ions generated in the ion source are rapidly accelerated to the same high-kinetic energy by a high voltage (V) of up to 30 kV and expelled into a field free drift tube with a length *L*, typically 0.5-2.0 m where they physically separate from each other based on their mass-to-charge (m/z) ratios. The detector records the arrival time and intensity of signals as the groups of mass-resolved

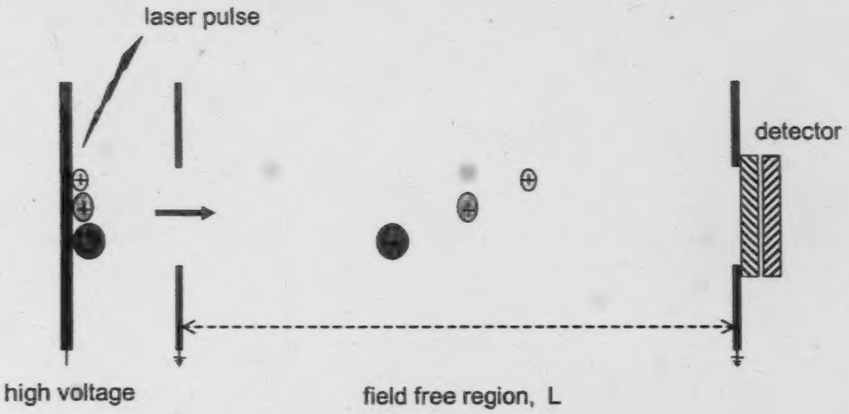

**Figure 1.5** Schematic of a linear TOF mass spectrometer.

ions exit the flight-tube. The equations to describe the basic principle, with the approximation that no ions possess initial kinetic energy are listed below (Equations 1.1-1.3). The flight time, $t$, depends upon the velocity, $v$, and therefore, depends upon m/z as well.

$$ZeV = \frac{1}{2}mv^2$$ **Equation 1.1**

$$t = L / v$$ **Equation 1.2**

$$v = (\frac{2ZeV}{m})^{0.5} \Rightarrow t = (\frac{m}{Z})^{0.5} \frac{L}{(2eV)^{0.5}}$$ **Equation 1.3**

in which $e$ is the unit of elementary charge, $m$ is the mass of the ion, $Z$ is the charge state, $v$ is the linear velocity, and $L$ is the flight path length.

For a linear TOF analyzer, the mass resolution is usually poor (< 300 fwhm) due to the broad initial kinetic energy, spatial and temporal distributions of the ions formed by MALDI. Time-lag focusing and/or a reflectron [82-83] are used to improve mass resolution and mass accuracy of MALDI-TOF instrument.

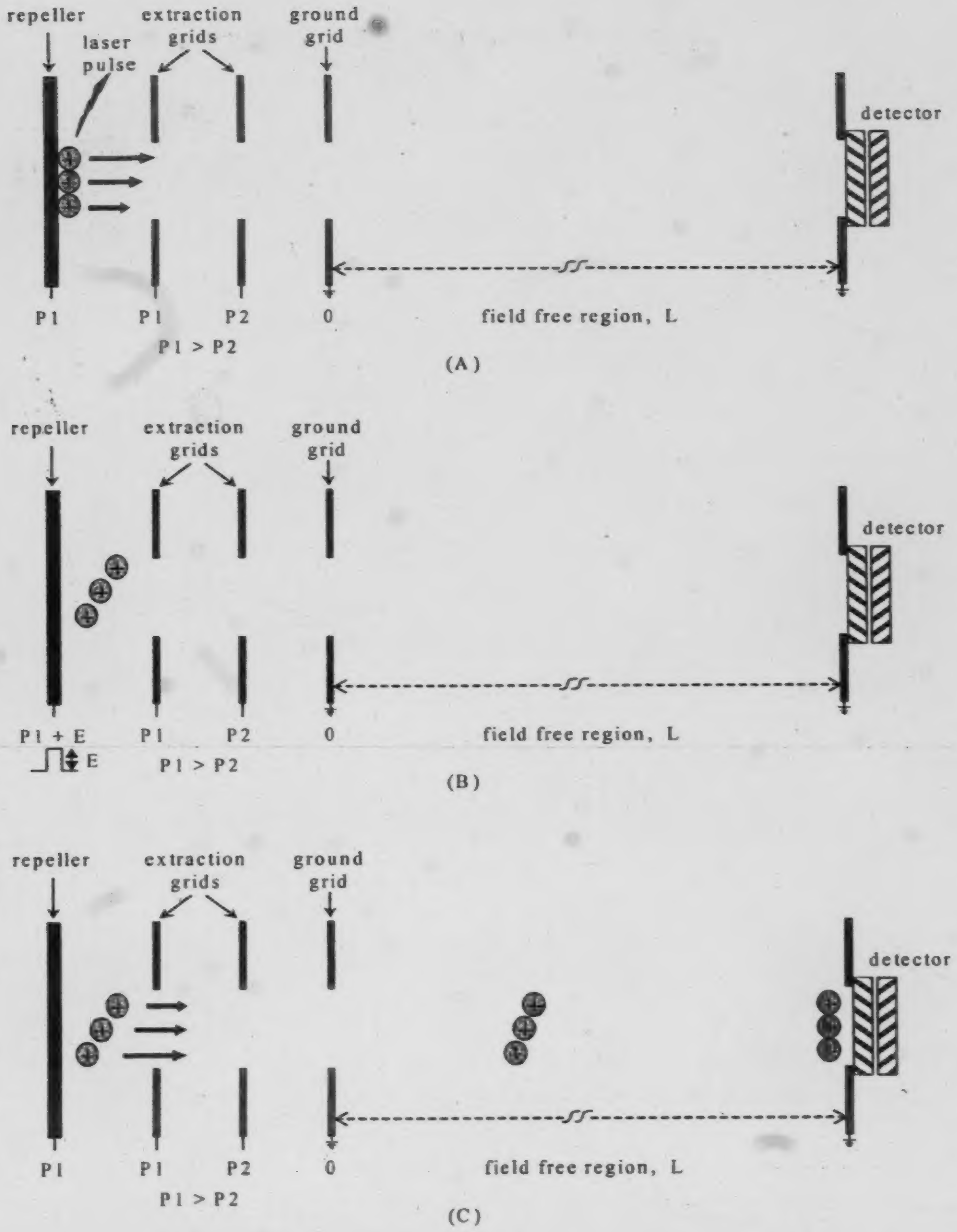
repeller
laser pulse
extraction grids
ground grid
detector
P1
P1
P2
0
field free region, L
P1 > P2
(A)
repeller
extraction grids
ground grid
detector
P1 + E
E
P1
P2
0
field free region, L
P1 > P2
(B)
repeller
extraction grids
ground grid
detector
P1
P1
P2
0
field free region, L
P1 > P2
(C)

**Figure 1.6** Schematic of time-lag focusing in a linear TOF MS. (A) Ions with same *m/z* but different initial kinetic energy expand away from the repeller. (B) Ions with higher energy move further away from the repeller that is applied to an extraction pulse after a certain time delay. (C) Ions reach the detector due to the energy compensation.

In time-lag focusing MALDI-TOF (see Figure 1.6), ions are first allowed to expand into a field-free region between the repeller and the first extraction grid that are applied the same high voltage (Figure 1.6A). In this region, ions of the same m/z with different initial axial velocities will expand away from the repeller different distances. After a certain time lag, a dc voltage pulse is applied to the repeller to "kick" the ions into the flight tube (Figure 1.6B). This pulse imparts more energy to the ions closer to the repeller than those of the same m/z but further from the repeller. Therefore, ions of the same m/z with the initial axial velocity distribution can reach the detector at the same time such that peak broadening will be greatly reduced (Figure 1.6C).

Mass resolution in MALDI TOF-MS can also be achieved by the utilization of a reflectron, which is made of a number of electric plates with electric potential gradient. The ions of higher kinetic energy penetrate deeper into the reflectron and take a longer time to return. With properly arranged geometry and voltages, those ions of identical mass but different initial kinetic energy will reach the detector at the same time.

MALDI-TOF instrument combining time-lag focusing with a reflectron can achieve mass resolution of 10,000-20,000 fwhm [83]. In our work, a reflectron time-lag focusing system is always applied to peptides for its high resolution and high mass accuracy (better than 500 ppm or 0.05%) . For protein analysis, a linear time-lag focusing MALDI-TOF configuration is often used due to the sensitivity consideration.

### 1.3.2 ESI Quadrupole MS

ESI is an ionization method used to produce gaseous ionized molecules from a liquid solution at atmospheric pressure. Electrospray was first proposed by Dole and co-workers [84] in 1968 for detecting polymeric species such as the polystyrenes. This technique has been developed to an important method for the analysis of biomolecules such as protein and peptide [85-86], since Fenn and co-workers [87-89] successfully coupled it to a mass spectrometer in 1984. Figure 1.7 shows the principle of the electrospray ionization process [90]. At the onset of the electrospray process, a dilute solution of the analyte is injected at a constant lower flow rate through a small diameter

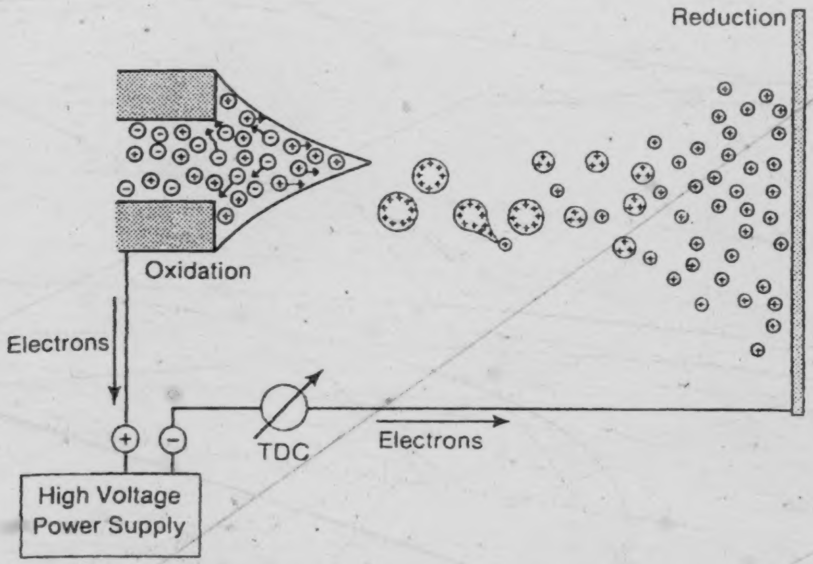

**Figure 1.7** Principle of the electrospray ionization process.

capillary or needle tip that is applied to a high voltage (3-5 kV) relative to a counter electrode. This high potential on the liquid creates a Taylor cone at the exit. A thin liquid extends from this cone, which breaks into a mist of fine charged droplets. Then these charged droplets shrink due to evaporation of the solvent by flowing a dry nitrogen gas at moderate temperature. As the droplets shrink, coulombic repulsion forces become sufficient to overcome the surface tension, and the charged droplets disintegrate into fine droplets. The shrinking and disintegrating process keep repeating until small enough droplets are formed. At this time, the electric field at the liquid surface becomes so high that the solute ions "escape" from the liquid phase to the gas phase (ion-evaporation). Subsequently the ions are attracted into mass analyzer to become detectable [90].

Since ESI generates ions continuously, it is often coupled to a scanning mass analyzer such as a quadrupole (Q) or a quadrupole ion trap (IT) that separate the ions based on their mass/charge ratio. The operation of both Q and IT is based upon the motion of ions in an oscillating electric field. Only the quadrupole mass analyzer is discussed in the following since it was used to analyze the extract from bacterial cells in this work.

A quadrupole mass analyzer, or a quadrupole mass filter, consists of four high-precision cylindrical rods located precisely in an orthogonal array. In this device (Figure 1.8), diametrically opposing rod pairs are electronically connected to form a positive pair

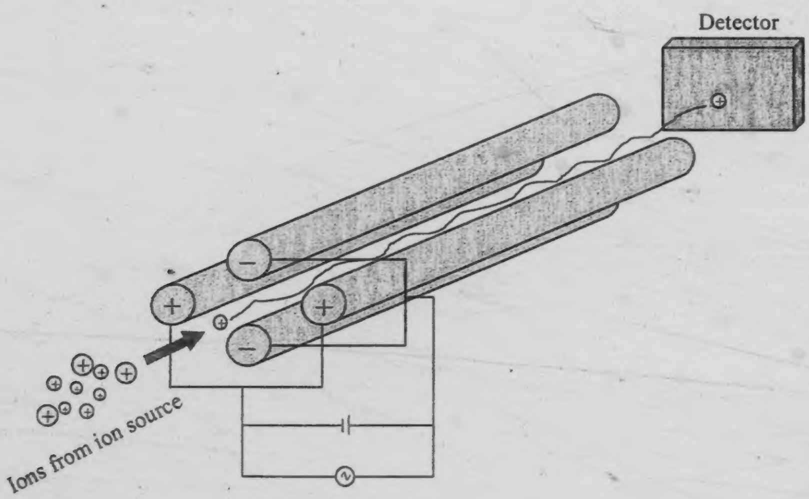

**Figure 1.8** Schematic of a quadrupole ion trap mass spectrometer.

and a negative pail of rods. The positive pair has a RF voltage superimposed on a positive DC voltage, while the negative pair has a negative DC and RF voltage that is 180 degrees out of phase with the positive pair. By adjusting the potentials, only ions within a small mass window can get through while all others are neutralized by one set of rods or the other. Ions of different *m/z* are transmitted through the analyzer from low to high mass by simultaneously increasing the values of U and V while keeping their rations constant.

Quadrupole mass analyzer in conjunction with ESI sources has two main advantages. First, the pressure in the analyzer can be as high as ~5 x $10^{-5}$ torr, which makes it well-suited to electrospray ionization in which the ions are produced at atmospheric pressure. Second, this configuration can be readily coupled to liquid chromatography.

### 1.4 A Brief Summary of This Thesis

In this thesis, the protocol to generate an experimentally derived protein mass database for bacterial identification is first established and described in Chapter 2. Based on this protocol, a model of experimentally derived database is then created and discussed in detail in Chapter 3. This database has included nine bacteria species, namely *Eschericha coli* (*E. Coli*), *Bacillus globigii*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus arlettae*, *Staphylococcus kloosii*, *Streptococcus bovis*, *Microbacterium saperdae* and *Pseudomonas fluorescens*. In Chapter 4, the low mass protein expression of bacteria cells as a function of cell culture, an issue related to the database creation, is discussed in detail. Finally, the reliability, applicability, and sensitivity of protein mass mapping approach combined with experimentally derived protein mass database was tested in Chapter 5.

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# Chapter 2

# Strategies of Creating Experimentally-Derived Protein Mass Database for Bacterial Identification by Mass Spectrometry

## 2.1 Introduction

The methods for MS-based bacterial identification have been extensively exploited in recent years. Initially, the method was largely relied on the comparison of a mass spectral profile from an unknown sample with the archived sets obtained from known bacteria [1-10]. However, the application of this approach was limited by the irreproducibility of mass spectra [3, 11-13]. An approach of bacterial identification by protein database search was thus emerged and studied [14]. In this approach, the protein masses in the spectrum of an unknown sample were basically matched with an internet-accessible protein database instead of a mass spectral library. Even though this approach brought a new concept to analytical chemists, it still needs further modifications to overcome the drawbacks originating from the public proteome database. Upon this consideration, an alternative novel approach has been proposed to improve both the accuracy and reliability of searched databases. In this method, the set of protein masses in a mass spectrum of an unknown species were matched with an experimentally derived protein mass database including many known bacteria [15]. This method has some advantages over the other protein-biomarker-based ones. Especially, as the detail experimental factors are taken into account during the identification and discrimination process, the inaccuracy and uncertainty caused by some varying factors such as ecological environment, growth time, pH value, and temperature are thereby minimized.

To test and apply this new approach, it is imperative to first establish a general protocol for the creation of experimentally derived protein mass database used in this thesis. This database shall contain a partial list of protein masses for a variety of known bacteria detectable by the mass spectrometry techniques we employed. It is also important that the creation and compilation of such a database should be conducted on a sound and logical ground; otherwise, better accuracy and reliability for identification may not be attained. Finally, the database should facilitate our future work on devising a suitable statistical matching model and eventually realizing computerized automation of bacterial identification. In this chapter, strategies for creating an experimentally derived mass database have been tested and are described in the following context.

## 2.2 Experimental

### 2.2.1 Chemicals and Materials

*Escherichia coli* (*E. coli*) (ATCC 9637) was grown in our lab from a stock of lyophilized cells of Strain W (ATCC 9637) purchased from Sigma Aldrich Canada (Oakville, ON). It was grown in Luria Broth (LB) (BBL, Becton Dickinson) at 37 °C with constant shaking. The cells were harvested at 8, 9, 10, 12, 16, 20, 24, 36 and 48 h, and were subsequently washed with MQ water once, lyophilized and stored at –25 °C until analysis.

Luria Broth was from Becton Dickinson Biosciences (Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich-

Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile and glacial acetic were from Fisher Scientific Canada (Edmonton, AB).

### 2.2.2 Sample Preparation

For each of the 9 incubation times examined, *ca.* 6 mg of lyophilized cells was suspended in 1 mL of 0.1% (v/v) TFA. The mixture was cooled with an ice-water bath and sonicated with a Branson probe sonicator. The probe tip of the sonication unit was immersed in the solution and the sonication was conducted for 1 min (pulsed mode: 1 pulse per second, pulse duration: 0.75 seconds). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 2.2.3 Mass Spectrometry

**LC/ESI-quadrupole.** Solvent delivery and separations were performed on an Agilent (Palo Alto, CA) HP1100 HPLC system. Chromatographic analysis was performed with a reversed-phase Vydac $C_8$ column (300Å, 5μm, 2.1mm i.d. × 150mm). The mobile phase consisted of $H_2O$ and acetonitrile, both with 0.05% (v/v) TFA. Gradient elution at 200 μL/min was employed using the following times and % acetonitrile (min:%ACN) 0:2, 10:20, 40:40, 45:55, 60:90, 65:2. Post-column glacial acetic acid was infused at 100 μL/min by a polyetheretherkrtone (PEEK) "Y" connector and syringe pump (Cole Parmer, Vernon Hills, IL). The HPLC effluent was analyzed with an Agilent 1100 series MSD quadrupole mass spectrometer. A variable fragmentation voltage was used during the ESI detection. The optimized voltage ramp was found to be at *m/z* 500, 60 V; at *m/z* 1000, 120 V; at *m/z* 3000, 220 V.

## 2.3 Results and Discussion

### 2.3.1 Experimental Conditions for Bacterial Protein Expression

In the process of creating the protein mass database, a major consideration is on the selection of experimental conditions to be used for bacterial protein expression. A desirable goal is to select conditions under which protein expressions reflect the major changes associated with microbiological variations. This is critical for the success in applying the database for identification of unknown bacteria that are produced under different growth time and growth culture conditions.

To create the database, we have chosen to vary the cell growth times and determine the masses of the expressed proteins by MS at each growth time. Arnold *et al.* reported that the matrix-assisted laser desorption ionization (MALDI) mass spectra obtained from whole cells of *E. coli* strain K-12 harvested at different growth times varied considerably in the appearance of mass values and intensities of peaks [11]. This example reinforces that the proteome information of a bacterial cell necessarily depends on the growth time. In our work, to probe the relationship of protein expression with growth time and its corresponding reflection in MS analysis, *E. coli* (ATCC 9637) was investigated as a model. *E. coli* (ATCC 9637) was grown from a stock of lyophilized cells and was harvested at nine different times between 8 and 48 h. The singly protonated protein ions from the nine different growth times were determined by high performance liquid chromatography (HPLC) electrospray ionization (ESI) Mass Spectrometry (MS) and were listed in Table A.1 (see Appendix). The distribution of mass entries observed in the samples collected at all incubation times was shown in

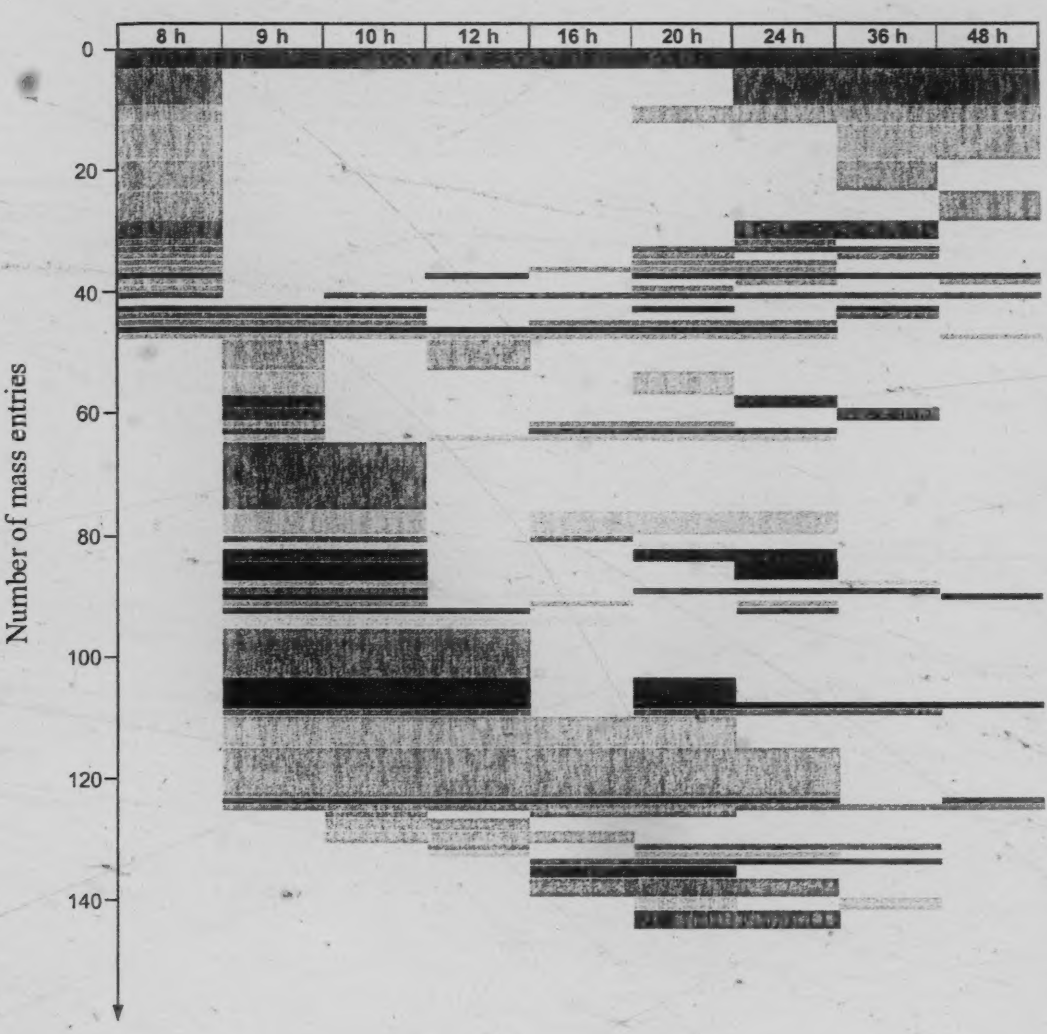


**Figure 2.1** Distribution of mass entries obtained from *E. coli* (ATCC 9637) extracts by LC/ESI-MS.

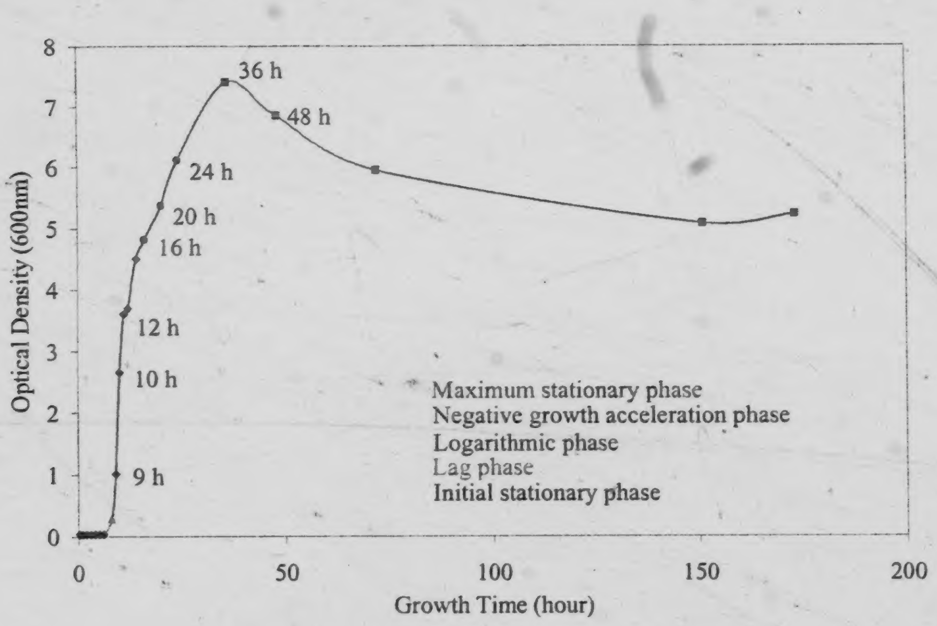


**Figure 2.2** Growth cure for *E. coli* (ATCC 9637).

Figure 2.1. In Figure 2.1, the horizontal position of a strip indicates the growth time when a mass entry can be detected from the harvested sample. The vertical length of a strip indicates the number of mass entries that can be observed in the same growth periods. From Figure 2.1, it can be seen that mass data pattern from the samples obtained from 9 h to 24 h overlapped. In this period (see Figure 2.2), there were enough nutrients in the culture. The cell number increased quickly. The environmental conditions did not changed very much. So protein expressions associated with the growth conditions did not vary dramatically with the growth time. The mass entries observed in the samples collected at 10 h, 16 h and 20 h can also be found at those harvested at 9 h, 12 h and 24 h. Even though we do not take samples at 10 h, 16 h, and 20 h, we still can obtain protein expression information of these three growth time samples from the samples harvested at growth time around them. This study suggests that an optimal approach of detecting as

many different proteins as possible is to harvest and analyze each bacterial species several times from different growth phases according to their respective cell growth curves.

For each species, mass data obtained from various growth periods were compiled to give a protein mass table, and the set of these tables from many different known bacteria comprised the experimentally derived protein mass database. In this way, the microbiological factor of varying growth times is coherently included in the database.

### 2.3.2 Sample Preparation for Bacterial Protein Expression

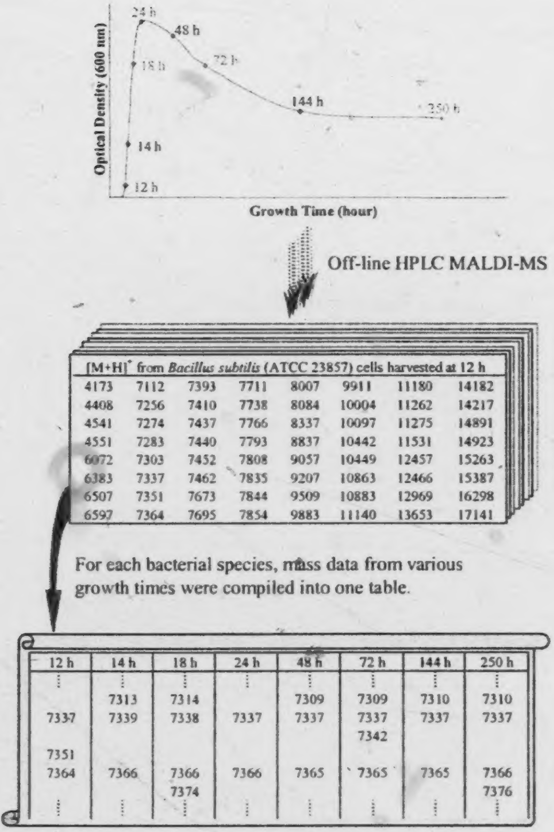
The database for the bacterial identification should ideally consist of protein entries from all bacteria of interest and include all proteins that can be expressed for a given bacterium. However, in practice, such database cannot be constructed with a very high throughput for some reasons. First, many proteins are not highly expressed or readily detected by current analytical technologies. Even though protein mixture can be separate by HPLC or 1D or 2D PAGE, ion suppression still is an issue under investigation. Second, the molecular masses of prokaryotic proteins distribute in a broad range from several kDa to one hundred kDa with a distribution in which the center peak is around 12 kDa. Mass spectrometer usually just favourites the detection of lower molecular weight proteins. Especially, the detection range for MALDI is below 30 kDa. However, low mass range proteins (2-20 kDa) can be used for microorganism identification [14]. Third, p*I* values of bacterial proteins are mainly between 2 and 13. Some proteins are basic, while others are acidic. The protein extract solution that is basic or acidic only can favor the detection of parts of proteins. Finally, the value of hydrophobicity of each protein is different. Usually hydrophilic proteins are more soluble in the water solution, which make it easier to be detected.

Proteins can be detected from cell extract [1-4, 16-22] or whole cells directly [5-8, 11-13, 23-27]. However, an inadequate quantity of proteins usually results from whole-cell Gram-positive bacteria due to their thick cell walls [28]. Since a large number of protein entries for each bacterial species in a database are crucial for reliable and effective bacterial identification [15], we adopted a protocol for the generation of a sufficient number of protein masses from cell extract. Specifically, water-soluble low mass proteins (typically at the mass range from 2-20 kDa) from each bacterial species will be detected and included in the database.

### 2.3.3 Detection of Bacterial Protein

Another consideration in the creation of an experimentally derived protein mass database is the mass spectrometric technology to be used for protein mass analysis. It is of vital importance to determine masses for as many proteins as possible from each bacterium of interest with high throughput. This will ensure the inclusion of a large fraction of the proteome of a given bacterium in the database and the rapid creation of the database to include the bacteria of interest for specific applications.

Protein mass analysis in creating the protein mass database was done by using a newly developed HPLC interface with off-line MALDI time-of-flight (TOF) MS. Previously, we have demonstrated that ion suppression can be moderated when cell extracts are subject to HPLC prior to MS detection in the analysis of a complex protein mixture [4]. In this way, more protein masses were detected by the combination of HPLC with ESI- or MALDI-MS. ESI-MS easily interfaces in an on-line fashion with



Off-line HPLC MALDI-MS

| $[M+H]^+$ from *Bacillus subtilis* (ATCC 23857) cells harvested at 12 h | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4173 | 7112 | 7393 | 7711 | 8007 | 9911 | 11180 | 14182 |
| 4408 | 7256 | 7410 | 7738 | 8084 | 10004 | 11262 | 14217 |
| 4541 | 7274 | 7437 | 7766 | 8337 | 10097 | 11275 | 14891 |
| 4551 | 7283 | 7440 | 7793 | 8837 | 10442 | 11531 | 14923 |
| 6072 | 7303 | 7452 | 7808 | 9057 | 10449 | 12457 | 15263 |
| 6383 | 7337 | 7462 | 7835 | 9207 | 10863 | 12466 | 15387 |
| 6507 | 7351 | 7673 | 7844 | 9509 | 10883 | 12969 | 16298 |
| 6597 | 7364 | 7695 | 7854 | 9883 | 11140 | 13653 | 17141 |

For each bacterial species, mass data from various growth times were compiled into one table.

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |
| | 7313 | 7314 | | 7309 | 7309 | 7310 | 7310 |
| 7337 | 7339 | 7338 | 7337 | 7337 | 7337 | 7337 | 7337 |
| | | | | | 7342 | | |
| 7351 | | | | | | | |
| 7364 | 7366 | 7366 | 7366 | 7365 | 7365 | 7365 | 7366 |
| | | 7374 | | | | | 7376 |
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |

**Figure 2.3** Procedures involved in creation of protein mass database tailored for bacterial identification by protein mass mapping.

HPLC and is routinely used in the characterization and determination of protein mixtures. However, MALDI-MS combined with the two-layer sample/matrix preparation method is advantageous in the detection of proteins from lysed bacterial samples because of its simplicity for data processing, high mass accuracy, high sensitivity, and high resolution [29, 30]. In addition, by using off-line HPLC MALDI-MS, more components in the cell extract fraction can be obtained compared to HPLC ESI-MS, because the concentration step in the off-line MALDI analysis provides a much higher amount of protein in each fraction than that in the HPLC ESI-MS analysis [15]. In our off-line HPLC MALDI-MS method, HPLC fractions can be concentrated on the MALDI target directly. As a result, the time-consuming concentration process and unwanted protein loss due to coagulation on the wall of a vial during concentration can be avoided.

## 2.4 Conclusions

In this chapter, the strategy for creating an experimentally derived mass database for bacterial identification is described. As summarized in Figure 2.3, the MS method for the creation of the protein mass database possesses the following features. For each bacterial species, water-soluble low mass protein data from protein extracts obtained from samples harvested at various growth periods were compiled into a protein mass table. The set of these tables from many different known bacteria then comprised the experimentally derived protein mass database. In principle, a database generated by the method described in this chapter shall display an improved accuracy and reliability, owing to its inherent advantages of: 1) it includes factors of various growth conditions; 2) there are sufficient protein entries with high mass accuracy for each bacterial species; and 3) it contains experimentally derived post-translational modification information.

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# Chapter 3

# Experimentally Derived Protein Mass Database by Mass Spectrometry[a]

Bacterial identification using an experimentally derived protein mass database [1] has been of great interest in our research group during the past years. To reach this goal, it is necessary to generate a practical and reliable experimentally derived protein mass database. The protocol to create such a database has already been established [see Chapter 2]. In this chapter, we will mainly focus on the description of experimental details related to the creation of the database.

Ideally, an experimentally derived database should include as many known bacterial species as possible. Due to the time constraint, we envisioned an approach of completing the database in steps. In other words, we first build up a prototype of database including a limited number of bacteria, and then gradually update the database by including more bacterial species of interest. In the meantime, tests on reliability and applicability of the database can be performed in parallel to the task of database expansion.

Accordingly, in our first prototype database, we selectively included only several bacterial species of interest to us. Generally, the Protophyta is divided into the following three classes: Schizophyceae, Schizomycetes that include bacteria, and microtatobiotes. There are ten orders in the class Schizomycetes, namely, Pseudomonadales,

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[a] Ms. Xinlei Yu and the author contributed equally to this work because of the bulk results of the data collected.

Eubacteriales, Actinomycetales, Spirochaetales, Mycoplasmatales *etc.* Among them, Eubacteriales is referred to as typical bacteria. Most bacteria that are of medical interest are in this order. Therefore, species *Escherichia coli*, *Bacillus globigii*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus arlettae*, *Staphylococcus kloosii*, *Streptococcus bovis*, and *Microbacterium saperdae* from the order Eubacteriales as targets were chosen. Species *Pseudomonas fluorescens* from the order Pseudomonadales were investigated as well since the genus *Pseudomonas* is important both scientifically and practically. To date, a preliminary experimentally derived bacterial protein mass database comprising *nine* species has been successfully generated. Specific descriptions of each bacterial species are also introduced in the following sections.

## 3.1 *Escherichieae coli*

### 3.1.1 Introduction

*Escherichia coli* (*E. coli*) is in the family of Enterobactericeae and is Gram-negative. In "*Bergey's Manual of Systematic Bacteriology*" it is described as:

> *Straight rods, 1.1-1.5 μm × 2.0-6.0 μm, occurring singly or in paris. Capsules or microcapsules occur in many strains. Motile by peritrichous flagella or nonmotile. Facultatively anaerobic, having both a respiratory and a fermentative type of metabolism* [2].

*E. coli* widely presents in all kinds of environment. In human body, it naturally lives in the intestinal tract and acts as the causative agent in pathologic processes in some instances. In this section, strain *E. coli* (ATCC 47076) has been analyzed to provide a protein mass table for species *E. coli*, because this strain has the most completed proteome database in the public domain.

### 3.1.2 Experimental

#### 3.1.2.1 Chemicals and Materials

*E. coli* (ATCC 47076) was grown in our lab from a stock of lyophilized cells (ATCC 47076) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding 40 mg of *E. coli* (ATCC 47076) into 50 mL of 1065 LB medium (10.0 g tryptone, 5.0 g yeast extract, and 10.0 g sodium chloride in 1.0 L distilled water). The culture was kept at 37 ° C for 2 h. Then this starter culture was added into 1550 mL of scale-up culture. After 2 h, a portion of 200 mL was transferred to a centrifuge tube and spun for 20 minutes. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 2 h. Same procedures were repeated at 4, 12, 24, 48, and 72 h respectively.

Tryptone and yeast extract were from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), formic acid, $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and sodium chloride were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile and methanol were from Fisher Scientific Canada (Edmonton, AB).

#### 3.1.2.2 Sample Preparation

For each of the 6 incubation times examined, *ca.* 6 mg of lyophilized cells was suspended in 1 mL of 0.1% (v/v) TFA. The mixture was cooled with an ice-water bath and sonicated with a Branson probe sonicator. The probe tip of the sonication unit was immersed in the solution and the sonication was conducted for 1 min (pulsed mode: 1

pulse per second, pulse duration: 0.75 seconds). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 3.1.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** Bacterial extracts from *E. coli* (ATCC 47076) were separated on a $C_8$ Vydac column (300 Å, 5 μm, 1.0 mm i.d. × 150 mm) at a flow rate of 40 μL/min. Solvent delivery and separations were performed on an Agilent (Palo Alto, CA) HP1100 HPLC system. The mobile phase consisted of $H_2O$ and acetonitrile, both containing 0.1 % (v/v) TFA. The gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 10:20, 40:40, 45: 55, 55:65, 65:90, and 70:2. HPLC fractions were collected on a 100 well MALDI target (Applied Biosystems) directly. MALDI experiments were performed using a Bruker REFLEX III matrix-assisted laser desorption/ionization time-of-flight mass (MALDI/TOF) spectrometer (Framingham, MA) on each individual fraction. Desorbed protein ions were produced with a 337 nm pulsed nitrogen laser and detected in the linear mode of operation in TOF. A pseudo-two-layer sample preparation method with HCCA as the matrix was used for MALDI analysis. In the pseudo-two-layer method, the first layer was formed by applying 0.8 μL of 0.1 M HCCA in methanol/acetone (3/7, v/v) to the MALDI plate. For the second layer, protein components collected on the 100 well MALDI target were redissolved with 1 μL of transfer solution consisted of 50% methanol and 50% saturated HCCA in formic acid/methanol/water (1:2:3, by volume). Then this mixture was applied onto the first layer and allowed to dry. External mass calibration was done using horse cytochrom *c* as a standard.

### 3.1.3 Results and Discussion

#### 3.1.3.1 Protein Extraction

Since a large number of protein database entries for a bacterial species are crucial for reliable and effective bacterial identification, the protein extraction is an important factor in the creation of experimentally derived protein mass database [1]. Cell disruption plays a significant role in releasing proteins from bacterial cells [3]. It is known that bacteria cells can be lysed by sonication more completely than simple vortexing [4]. Cells are disrupted by sonication force due to gaseous cavitation [5-8]. As a Gram-negative species, *E. coli* (ATCC 47076) has a relatively thin peptidoglycan layer (see Figure 1.2). Hence, it is easy to break the cell wall and obtain reasonable levels of protein by a probe-tip sonicator. Both acidic solution (0.1% TFA) [3, 4, 9-12] and basic solution (50 mM Tris-HCl buffer) [3, 13] can be used as a suspension solvent for protein extraction [3]. For *E. coli*, there are more low molecule weight proteins (< 20 kDa) in the 0.1% TFA extract, while higher molecule weight proteins (20-100 kDa) can be more efficiently extracted by 50 mM Tris-HCl buffer [14-15]. Usually, MALDI favorites low molecule weight proteins (< 30 kDa). Even though there are many protein components from the higher mass range in the extract, only those with low molecule weight can be detected in MALDI experiment. Moreover, some proteins may be lost due to precipitation during the acidification of protein extract in 50 mM Tris buffer (pH 8.5). In general, a protocol of sonication using 0.1% TFA solution for protein extraction from *E. coli* (ATCC 47076) cells was adopted in our experiments.

#### 3.1.3.2 Mass Tables

Protein extract from each sample harvested at different growth time was separated by HPLC. Each HPLC fraction was then detected by MALDI. Usually, several $[M+H]^+$ peaks can be obtained from one mass spectrum. However, not all of them were selected into the experimentally derived mass database. Only the peaks possessing the signal to noise ratio (S/N) above *three* were listed into a mass table. By doing so, we can ensure that the mass database is comprised of specific biomarkers for *E. coli* (ATCC 47076), rather than a collection of randomly detected mass entries resulting from the variation of sample preparation or ionization in MALDI.

In the MALDI analysis, clusters of peaks were sometimes observed. One of them is due to the detection of methionine-oxidized proteins in addition to their non-oxidized forms. Usually masses of the oxidized proteins were deleted from the table in order to reduce the probability of mismatching in the bacterial identification. For the same reason, peaks from $[M+Na]^+$and $[M+K]^+$ were also deleted.

Some protein components are eluted from the column at several fractions. Therefore they are detected several times in different spectra. The masses from the same protein components were averaged. These average masses were listed in the mass table. After creating one mass table from each sample collected at different growth time, those mass tables were compiled into one table for *E. coli* (ATCC 47076) (see Appendix, Table A.2). In the process of mergenee, masses in the range of ± 0.05% MW were considered as one mass entry and listed into the same row.

For *E. coli* (ATCC 47076) there are a total of 195 individual masses detected by LC/off-line MALDI over the entire incubation period. Table A.2 lists all of the $[M+H]^+$ values for each incubation time studied, whereas the bolded mass values represent those whose molecular masses do not match with the proteins in the SWISS-PORT or TrEMBL databases. As Table A.2 shows, many proteins detected by MS do not match with the proteome database in the public domain. Those un-matched protein entries indicate that the protein masses detected experimentally are very likely to be different from those derived from the genome due to post-translational modifications. Those matched mass entries may not stand for the same original proteins as those described in the public proteome database [16].

## 3.2 *Pseudomonas fluorescens*

### 3.2.1 Introduction

Genus *Pseudomonas* is in the family *Pseudomonadaceae*, which is in the order Pseudomonadales. It has been widely studied due to its scientific and practical importance. The pseudomonads are among the most active participants in the process of mineralization of organic matter in nature. Many members of the group include etiological agents of important disease of plants and animals. " *Bergey's Manual of Systematic Bacteriology* " describes it as:

> *Straight or slightly curved rods but not helical, 0.5-1.0 μm in diameter by 1.5-5.0 μm in length. Gram-negative. Motile by one or several polar flagella; rarely nonmotile. In some species lateral flagella of shorter wavelength may also be formed. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, allowing growth to occur anaerobically. Most species do not require organic growth factors. Widely distributed in nature* [17].

*Pseudomonas fluorescens* can be found in soil and water. It is probably the most complex species of the genus *Pseudomonas*. In this section, strain *Pseudomonas fluorescens* (ATCC 948) has been analyzed to provide a protein mass table for species *Pseudomonas fluorescens*.

### 3.2.2 Experimental

#### 3.2.2.1 Chemicals and Materials

*Pseudomonas fluorescens* (ATCC 948) was grown in our lab from a stock of lyophilized cells (ATCC 948) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding 1 pea-sized of frozen culture into 50 mL of nutrient broth. The culture was kept at 26 °C for 2 h. Then this starter culture was added into 1750 mL of scale-up culture. After 18 h, a portion of 300 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 18 h. The same procedure was repeated at 20, 24, 32, 48, 72, 120, 264, and 432 h, respectively.

Nutrient broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), formic acid, $\alpha$-cyano-4-hydroxycinamic acid (HCCA), and horse cytochrome *c* were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile and methanol were from Fisher Scientific Canada (Edmonton, AB).

#### 3.2.2.2 Sample Preparation

For each of the 9 incubation times examined, *ca.* 11 mg of lyophilized cells were suspended in 1.5 mL of 0.1% (v/v) TFA. The mixture was cooled with an ice-water bath and sonicated with a Branson probe sonicator. The probe tip of the sonication unit was immersed in the solution and the sonication was conducted for 1 min (pulsed mode: 1 pulse per second, pulse duration: 0.75 seconds). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 3.2.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.1.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:25, 30:35, 55:55, 60:80, and 65:2.

### 3.2.3 Results and Discussion

Since *Pseudomonas fluorescens* (ATCC 948) is also a Gram-negative bacterium, the protocol of sonication using 0.1% TFA solution was used for protein extraction. Protein extract from sample harvested at different growth time was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Pseudomonas fluorescens* (ATCC 948) (Appendix, Table A.3) are same as those described in Section 3.1.3.2.

For *Pseudomonas fluorescens* (ATCC 948), there are 186 individual masses in total detected by LC/off-line MALDI over the entire incubation period. Table A.3 lists all of the $[M+H]^+$ values for each incubation time studied. As shown in Table A.3, fewer protein entries were observed in shorter growth time (18 h and 20 h) than that detected in longer period (24 – 432 h). 2 kDa masses were observed only in the samples harvested from 48 to 432 h but in shorter growth time samples (18 – 32 h). Interestingly, high masses (26 kDa – 37 kDa) were detected in the samples of this bacterium. With external calibration, the mass measurement accuracy is expected to be better than 0.05%.

### 3.3 *Bacillus globigii*

Genus *Bacillus* is in the family *Bacillaceae*. Bacteria in this group are ubiquitous in nature, such as soil, water, and airborne dust *etc*. Some species are natural flora in the human intestines. A unique characteristic of this genus is its ability of forming endospores when environmental conditions are stressful. "*Bergey's Manual of Systematic Bacteriology*" describes it as:

> *Rod-shaped, straight or nearly so. Cells may occur sigly or in chains with considerable length. The rods may have rounded or aquared ends and may be quite small (0.5 μm × 1.2 μm) or rather large (2.5 μm × 10 μm) Usually Gram-positive. Most Bacillus species are motile by means of peritrichous flagella. Aerobic or facultatively anaerobic. Catalase formed by most species. The cell wall peptidoglycan of most species belongs to the directly cross-linked meso-diaminopimelic acid type* [18].

In the following sections, species of *Bacillus globigii*, *Bacillus subtilis*, and *Bacillus megaterium* are studied.

### 3.3.1 Introduction

*Bacillus globigii*, also known as *Bacillus subtilis*, is a bacterium that is not pathogenic and can be found in soils virtually everywhere. Recently, the analysis of *Bacillus globigii* has become a hot topic in the area of biological warfare detection since it is a surrogate species for highly pathogenic *Bacillus anthracis* [19-23]. Both of them are endospore-forming bacteria. The rapid identification of *Bacillus* spore using mass spectrometry has been investigated for almost a decade. Pyrolysis mass spectrometry (Py-MS) has been previously applied to the identification of spores by using dipicolinic acid, lipid, and carbohydrate as biomarker [19-22]. Spores were also investigated by direct probe mass spectrometry combined with electron ionization and chemical ionization where the peaks from DNA, fatty acids, and glycerides provided species-specific information [23]. With the development of soft ionization methods, the rapid characterization of *Bacillus* spores has been proposed using electrospray ionization [24-25] and matrix-assisted laser desorption/ionization mass spectrometry [27-28]. In those studies, not only small molecular components such as lipopeptide and lipophilic compounds but also some high-mass entires were employed as biomarkers. So far, relatively limited successes have been achieved in this respect because it is difficult to release sufficient number and amount of specific biomarkers from the multiple layers spores. Many groups use the direct analysis of whole cells [20, 22-23]. Therefore, only those compounds outside of spores could be detected as biomarkers in this approach.

In order to gain more specific information on these endospore-forming species, protein biomarkers from the spores were extracted and analyzed in our study. In this section, strain *Bacillus globigii* (ATCC 9372) was analyzed to provide a protein mass table for the species of *Bacillus globigii*.

### 3.3.2 Experimental

#### 3.3.2.1 Chemicals and Materials

*Bacillus globigii* (ATCC 9372) was grown in our lab from a stock of lyophilized cells (ATCC 9372) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding 40 mg of *Bacillus globigii* (ATCC 9372) into 50 mL of nutrient broth. The culture was kept at 30 °C for 2 h. Then this starter culture was added into 1550 mL of scale-up culture. After 9 h, a portion of 100 mL was transferred to a centrifuge tube and spun for 20 minutes. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 9 h. Sameprocedures were repeated at 11, 16, 24, 36, 48, and 72 h, respectively.

Nutrient broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), formic acid, *α*-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile and methanol were from Fisher Scientific Canada (Edmonton, AB).

#### 3.3.2.2 Sample Preparation

For each of the 7 incubation times examined, *ca.* 3 mg of lyophilized bacteria was weighed into a centrifuge tube and mixed with 800 µL of 50 mM Tris buffer (pH 8.5). The mixture was cooled with an ice-water bath and sonicated with a Branson probe sonicator. The probe tip of the sonication unit was immersed in the solution and the sonication was conducted for 15 min (pulsed mode: 1 pulse per second, pulse duration: 0.75 seconds). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

#### 3.3.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.1.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min:

%ACN) 0:2, 2:2, 15:25, 60: 55, 70:80, and 90:2. In the sample preparation for MALDI analysis, 1 μL of matrix solution that consisted of saturated HCCA in formic acid/methanol/water (1:2:3, by volume) was applied to the well of MALDI target on which protein components collected. Then this solution was allowed to dry.

### 3.3.3 Results and Discussion

#### 3.3.3.1 Protein Extraction

There are several methods used for spore cell lysis in laboratory, which are: 1) Freeze/thaw three or more times. But this treatment has proven to be inefficient. 2) Enzymes such as lysozyme treatment. However, this method may introduce non-specific biomarkers. 3) Corona plasma discharge (CPD). This method is mainly applied to the spores deposited on the MALDI target. 4) Sonication in a static chamber. This method is still not very efficient on spore disruption and requires lysozyme pretreatment or the addition of glass beads to improve the disruption efficiency [29-31]. Along the same line, a countinuous-flow ultrasound spore disruptor without any pretreatment has been reported [32]. 5) Probe-tip sonicator. This method has been used to successfully release protein biomarkers by Fenselau group [33]. Based on the reference of the manual for the sonicator, a probe-tip sonication for 15 min was used to extract proteins from spores in our study.

Both acidic and basic solutions were proposed as spore suspension solution [19-33]. However, the amount of protein extracted from spore cells using acidic solution was so low that no protein assay result could be obtained. This might be associated with the p*I* distribution of the proteins from the cells. As shown in Figure 3.1, most proteins from *Bacillus subtilis* cells are acidic (p*I* values were calculated form the SwissProt/TrEMBL

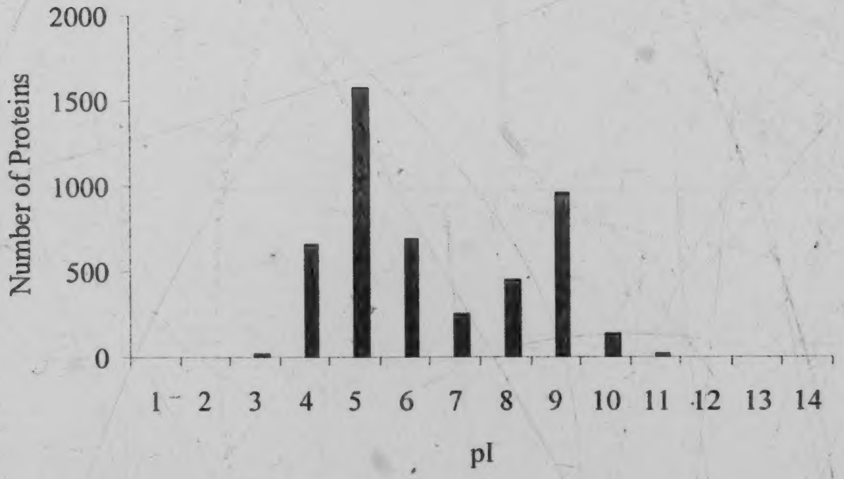

**Figure3.1** Distribution of p*I* values of *Bacillus subtilis* proteins.

database). Therefore, basic conditions of the cell suspension favor the protein extraction from Gram-positive cells.

One notable difference between our spore suspension solution and a typical lysing buffer is the absence of protease inhibitor and detergent in our system. Protease inhibitors are molecules that inhibit the activity of proteases in complex solutions such as cell lysates in order to preserve the native state of proteins. However, in our approach, so long as the mass entries listed in the experimentally derived mass database are characteristic for the corresponding bacterial species (i.e. biomarker), it is not a prerequisite they originate from the intact proteins. The addition of detergent in the cell suspension solution is to increase the solubility of proteins in the cell debris. However, such detergent as sodium dodecyl sulfate (SDS) are notorious for MS analysis due to the formation of multi-stage protein-sodium adducts [34]. Our approach, on the other hand, will primarily target on those soluble low mass bacterial proteins (2-30 kDa). Moreover, the most common practice in fast bacterial identification using mass spectrometry often involves a quick extraction using votexing with a simple solution followed by MS analysis. To keep consistent with the application condition, it seems sensible to not include protease inhibitor or detergent in the extraction solution during the creation a mass table. In this section, proteins from *Bacillus globigii* (ATCC 9372) cells were extracted with sonication for 15 min using 50 mM Tris buffer (pH 8.5).

#### 3.3.3.2 Mass Tables

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction collected on the MALDI target was directly detected by MALDI after treated with matrix solution in order to obtained more biomarkers for spore-forming species. The selection and exclusion of mass peaks and the creation of mass table for *Bacillus globigii* (ATCC 9372) (Appendix, Table A.4) are same as those described in Section 3.1.3.2.

For this bacterium, there are 390 individual masses in total detected by LC/off-line MALDI over the entire incubation period. Table A.4 lists all of the $[M+H]^+$ values for each incubation time studied. As shown in Table A.4, many low masses, especially in the mass range from 2 to 3 kDa, were detected in the sample. Less protein entries with high molecular weight (> 9 kDa) were observed in the samples harvested at 11 and 72 h than that detected in other period (16 – 48 h). With external calibration, the mass measurement accuracy is expected to be better than 0.05%.

## 3.4 *Bacillus subtilis*

### 3.4.1 Introdution

*Bacillus subtilis* is a widespread nonpathogenic spore-forming bacterium and the best-characterized member of the Gram-positive bacteria. It is commonly found in the soil, water sources, and in association with plants. The whole genome of *Bacillus subtilis* (strain 168) has already been sequenced. The sequence offers significant insights into the bacterium's capacity to use a wide variety of carbon sources and to secrete large quantities of industrially important enzymes. In this section, strain *Bacillus subtilius* (ATCC 23857) has been analyzed to provide a protein mass table for species *Bacillus subtilius*.

### 3.4.2 Experimental

#### 3.4.2.1 Chemicals and Materials

*Bacillus subtilius* (ATCC 23857) was grown in our lab from a stock of lyophilized cells (ATCC 23857) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding 1 pea-sized of frozen culture into 50 ml of potato extract medium (23.0 g nutrient broth 20.0 mL potato extract in 1.0 L distilled water). The culture was kept at 26 °C for 2 h. Then this starter culture was added into 1700 mL of scale-up culture. After 12 h, a portion of 300 mL was transferred to a centrifuge tube and spun for 20 minutes. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 12 h. Same procedures were repeated at 14, 18, 24, 48, 72, 144, and 250 h, respectively.

Nutrient broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), *α*-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from

Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

### 3.4.2.2 Sample Preparation

For each of the 8 incubation times examined, *ca.* 10 mg of lyophilized cells were resuspened in 4 mL of MQ water. The cell suspension was passed through a Thermo Spectronic French pressure cell at 18,000 psi, and the pH of the suspension was adjusted to < 2 with 10 % TFA. After centrifuging, the precipitate was mixed with 1 mL of 50 mM Tris-HCl buffer (pH 8.5). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 3.4.2.3 Total Protein Determination

The Tris extracts were mixed with Coomassie plus protein assay reagent and the absorbance was read at 590 nm. A standard curve was prepared using bovine gamma globulin (Pierce).

### 3.4.2.4 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.1.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:25, 60:55, 70:80, and 80:2. In the second layer preparation for MALDI analysis, protein components collected on the 100 well MALDI target were resolved with 1 μL of transfer solution which consisted of 50% acetonitrile and 50% saturated HCCA in acetonitrile/0.1% TFA (1:1, by volume). Afterward, this mixture was applied on the first layer and allowed to dry.

### 3.4.3 Results and Discussion

#### 3.4.3.1 Protein Extraction

Since both *Bacillus subtilius* (ATCC 23857) and *Bacillus globigii* (ATCC 9372) are Gram-positive bacteria, the same protocol as that for *Bacillus globigii* (ATCC 9372) cells (sonication for 10 min using 50 mM Tris buffer (pH 8.5)) was initially used to extract proteins from *Bacillus subtilius* (ATCC 23857) cells. Figure 3.2 shows the chromatogram of the protein extract from the cells harvested at 250 h. From Figure 3.2, it can be seen that there were a bunch of very broad peaks between 20 min (24% ACN) and 40 min (41% ACN); while nothing was eluted in the normal protein elution gradient range (45% to 80% ACN). However, the protein assay result (1,575 μg from 11 mg lyophilized cells) and Figure 3.3(A) indicate that sufficient proteins were indeed extracted using sonication with 50 mM Tris buffer. As pH value of protein extract was adjusted from 8.5 to 2.5 with 10% TFA before it was injected to the column, white precipitates were observed. Figure 3.3(B) is the MALDI spectrum of the supernatant after the acidification of protein extract. There are still proteins present in the supernatant even after precipitation. Interestingly, none of these proteins could be detected after HPLC. It is speculated that some components (we arbitrarily term them as "interference" component) in the cell extract possibly degrade the column performance. To gauge the origin of these components, French Press was used to disrupt the cells as an alternative. *Bacillus subtilius* (ATCC 23857) cells were resuspended in MQ water. Proteins were extracted with French Press. The pH value of suspension was adjusted to 2.5 and 8.5 with 10% TFA and 50 mM Tris, respectively. After centrifuging, supernatants with both

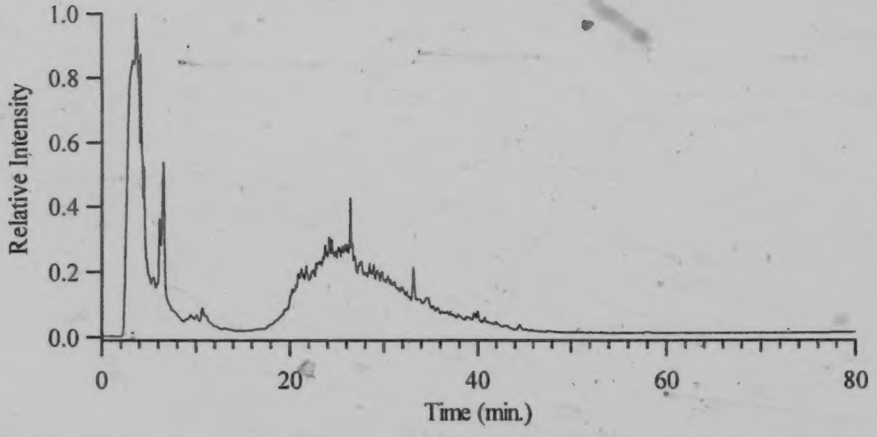

**Figure 3.2** Chromatogram of *Bacillus subtilis* (ATCC 23857) extract in 50 mM Tris using probe tip sonication from lyophilized cells harvested at 250 h.

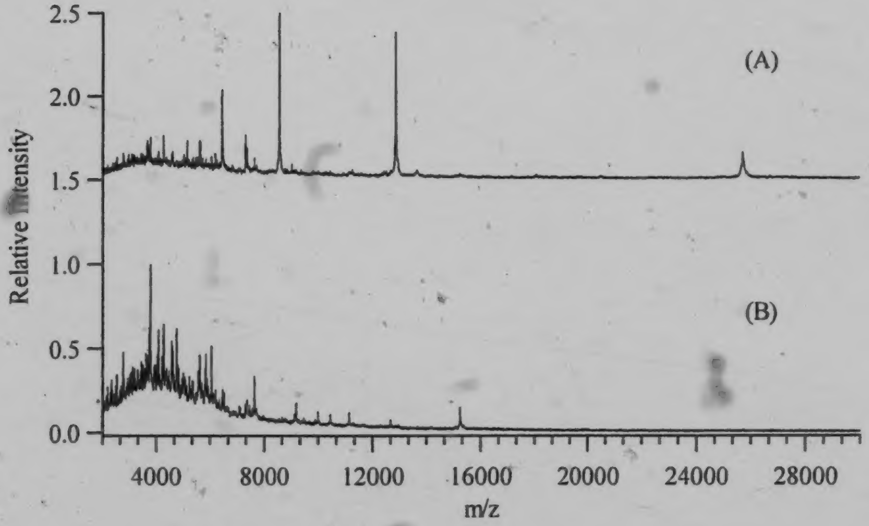

**Figure 3.3** MALDI spectra of: (A) *Bacillus subtilis* (ATCC 23857) extract using probe tip sonication in 50 mM Tris. (B) after the acidification.

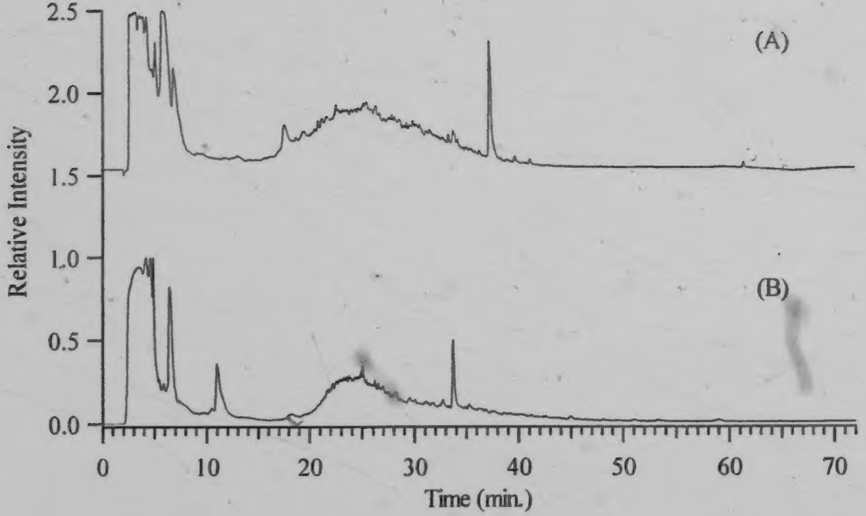

**Figure 3.4** Chromatograms of *Bacillus subtilis* (ATCC 23857) extracts using French Press from lyophilized cells harvested at 250 h. (A) acidic suspension. (B) basic suspension.

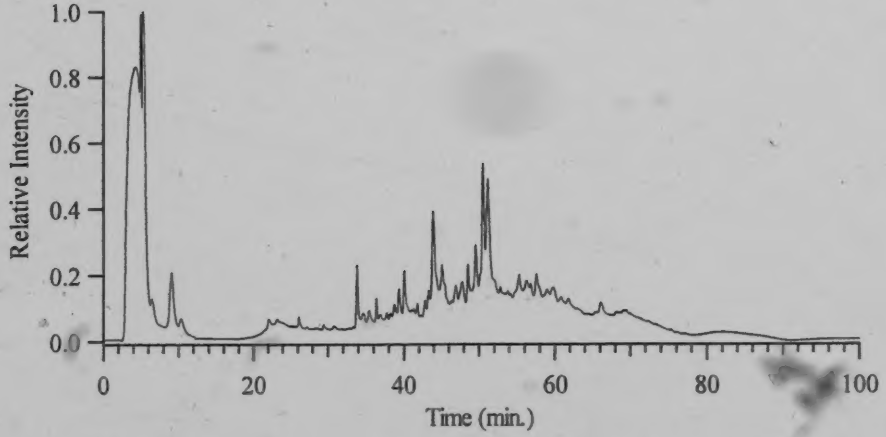

**Figure 3.5** Chromatogram of *Bacillus subtilis* (ATCC 23857) extract without "interference" components from lyophilized cells harvested at 250 h.

pH values were analyzed by HPLC. It has to be pointed out that although French Press might have high extraction efficiency, as in the current case, it also has the detrimental effect on the subsequent MS analysis. The silicone lubricant used in the French Press apparatus can interfere with MS analysis, resulting in poor spectral quality.

In Figure 3.4, trace A shows the chromatogram of the protein extract from the acidic suspension, while the chromatogram of protein extract from the basic suspension is shown in trace B. It is clear that the components affecting the column performance are not externally introduced during the extraction process. Very likely, they originate from the cells themselves. They are irrelevant to the extraction method or the pH value of the cell suspension.

**Table 3.1** Protein components of acidic suspension from *Bacillus subtilis* (ATCC 23857) cells harvested at 250 h by LC/off-line MALDI.

| Retention Time (min) | $[M + H]^+$ | | |
|---|---|---|---|
| 18 | 2467^3* | | |
| 19 | 2466^4 | | |
| 21 | 2466^3 | | |
| 29 | 4989^3 | 5101^3 | 5945^8 |
| 30 | 7220^3 | 9137^15 | |
| 32 | 2467^6 | 3182^3 | 6850^3 |
| 33 | 2467^18 | | |
| 34 | 2466^18 | 3957^3 | |
| 35 | 2466^14 | 3181^3 | |
| 36 | 2466^8 | | |
| 37 | 2467^8 | | |
| 38 | 2467^13 | | |
| 39 | 2470^10 | | |
| 40 | 2467^16 | | |
| 42 | 3256^5 | | |
| 61 | 2467^12 | | |
| 62 | 2467^27 | | |
| 63 | 2468^23 | | |

* A^B: A denotes $[M+H]^+$, B denotes S/N.

**Table 3.2** $[M+H]^+$ (Da) from acidic suspension from *Bacillus subtilis* (ATCC 23857) cells harvested at 250 h by LC/off-line MALDI.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2467 | 2470 | 3182 | 3256 | 3957 | 4989 | 5101 | 5945 | 6850 | 7220 | 9137 |

The protein extract from the acidic suspension was further analyzed by HPLC/off-line MALDI-MS. Table 3.1 lists the components detected from each fraction. Non-redundant $[M+H]^+$ values from the acidic suspension were listed in Table 3.2. There are a total of eleven mass entries detected by HPLC/off-line MALDI-MS; among them, component with nominal mass of 2467 Da is the most dominant one and can be observed in most fractions.

In order to remove the "interference" component, the following steps were taken, making use of the differential solubility of the protein of interest and "interference" component under acidic conditions. Protein extraction from *Bacillus subtilius* (ATCC 23857) cells favors the basic cell suspension but precipitates out under acidic conditions. To the contrary, those "interference" components can stay in the acidic solution. The precipitate from the acidic suspension was further treated with 1 mL of 50 mM Tris-HCl buffer (pH 8.5). Proteins precipitating under acidic condition were recovered without the company of the "interference" components. The mixture was votexted for 5 min and centrifuged at 11,750 *g*. The supernatant was injected to the column. The proteins extracted from *Bacillus subtilius* (ATCC 23857) cells with the new protocol can be successfully analyzed by HPLC/MALDI-MS, as shown in Figure 3.5.

### 3.4.3.2 Mass Tables

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Bacillus subtilis* (ATCC 23857) (Table A.5) are same as those described in Section 3.1.3.2.

For this bacterium, a total of 269 individual masses were detected by LC/off-line MALDI over the entire incubation period. Table A.5 lists all of the $[M+H]^+$ values for each incubation time studied, whereas the bolded mass values represent those whose molecular masses do not match with the proteins in the SWISS-PORT or TrEMBL databases. As shown in Table A.5, many proteins detected by MS do not match the proteome database in the public domain. Fewer low masses (2-3 kDa) were observed in the samples of this bacterium than other bacteria we had worked on. No mass between 2 and 3 kDa was detected in the samples harvested at shorter growth time (12 and 14 h). It is interesting that fewer entries with high molecular weight (> 15 kDa) were obtained in the samples collected at shorter growth time (12 and 14 h) than those harvested at longer growth time (16-250 h). With external calibration, the mass measurement accuracy is expected to be 0.05%.

## 3.5 *Bacillus megaterium*

### 3.5.1 Introduction

*Bacillus megaterium* has relatively large cells within the genus Bacillus. Therefore, it is named as "mega." This bacterium, like all species of Bacillus, forms spores. The spores usually are found in soil. *Bacillus megaterium* can synthesize a capsule containing not only polypeptide but also polysaccharide. In this section, strain *Bacillus megaterium* (ATCC 14581) has been analyzed to provide a protein mass table for species *Bacillus megaterium*.

### 3.5.2 Experimental

### 3.5.2.1 Chemicals and Materials

*Bacillus megaterium* (ATCC 14581) was grown in our lab from a stock of lyophilized cells (ATCC 14581) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding a loop of working stock into 50 mL of Nutrient broth. The culture was kept at 30 °C overnight. An appropriate volume of starter culture was added into 1750 mL of scale-up culture. After 4 h, a portion of 150 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 4 h. This procedure was repeated, in the same manner, at 8, 12, 24, 48, 96, 168, and 336 h, respectively.

Nutrient broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

### 3.5.2.2 Sample Preparation

For each of the 8 incubation times examined, *ca.* 10 mg of lyophilized cells were resuspened in 4 mL of MQ water. The cell suspension was passed through the Thermo Spectronic French pressure cell at 18,000 psi. The pH value of the suspension was then adjusted to 8.5 with 5 M Tris buffer. After the suspension was centrifuged at 11,750 *g*, the supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

#### 3.5.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.4.2.3.

### 3.5.3 Results and Discussion

#### 3.5.3.1 Protein Extraction

Since *Bacillus megaterium* is a Gram-positive bacterium, French Press was used to extract the proteins. Both acidic and basic suspensions were centrifuged. The supernatant of basic suspension was kept for HPLC analysis (see Figure 3.6(A)). The precipitate of acidic suspension was mixed with 50 mM Tris-HCl buffer. After the mixture was centrifuged, the resulting supernatant was injected into the column (see Figure 3.6(B)). As shown in Figure 3.6, no “interference” component in the protein extract from *Bacillus megaterium* (ATCC 14581) cells was found. Most of proteins may

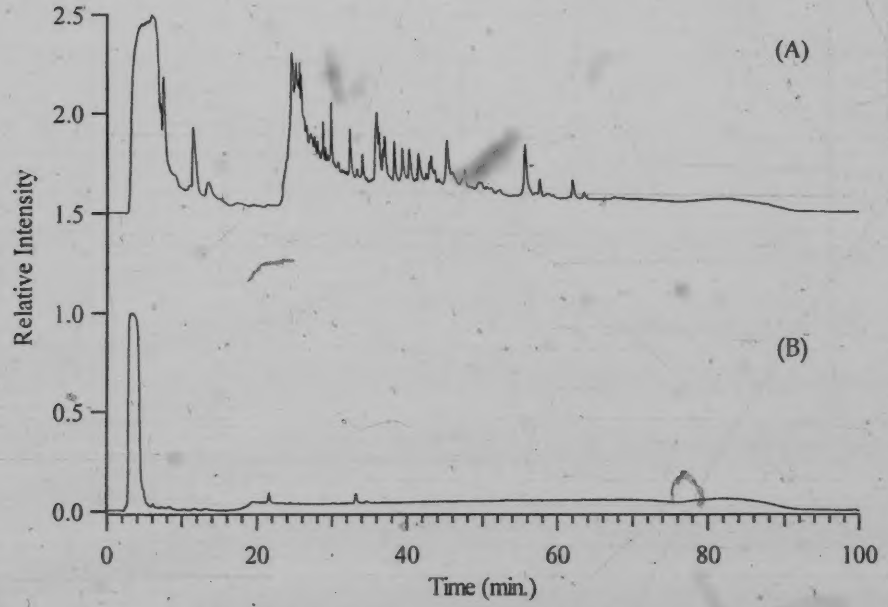

**Figure 3.6** Chromatograms of *Bacillus megaterium* (ATCC 14581) extracts from (A) the supernatant of basic suspension, (B) the precipitate of acidic suspension.

be lost if the special protocol for protein extraction from *Bacillus subtilis* (ATCC23857) cells was employed to *Bacillus megaterium* (ATCC 14581) cells. The normal protocol was then used for extracting *Bacillus megaterium* (ATCC 14581) cells.

### 3.5.3.2 Mass Tables

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Bacillus megaterium* (ATCC 14581) (see Appendix, Table A.6) are same as those described in Section 3.1.3.2.

For this bacterium, there are 386 individual masses in total detected by LC/off-line MALDI over the entire course of incubation. Table A.6 lists all of the $[M+H]^+$ values for each incubation time studied. With external calibration, the mass measurement accuracy is expected to be better than 0.05%. As shown in Table A.6, mass entries between 3 kDa and 5 kDa observed in the sample collected at 4 h were much less than those harvested at longer time (8–336 h). Higher mass entries (10 kDa–23 kDa) were mainly detected from the sample collected at 8–336 h. Moreover, mass entries above 20 kDa were only observed in the sample harvested after 96 h. The sample collected at 4 h possessed the minimal mass entry number among all the samples in this experiment.

## 3.6 *Staphylococcus arlettae*

### 3.6.1 Introduction

The genus *Staphylococcus* is the most important genus of the *Micrococcaceae* family in clinical area. It is phylogenetically unrelated to any other genera in the family even though *Staphylococci* are phylogenetically a coherent group of organism. On the basis of 16s RNA analysis, the genus *Staphylococcus* belongs to the broad *Bacillus*-

*Lactobacillus-Streptococcus* cluster. *Staphylococci* are non-motile Gram-positive bacteria [35]. The cells are spherical (0.5-1.5 μm in diameter). They grow in clusters like a bunch of grapes due to the fact that *Staphylococci* divide along two separate planes. The cell wall of *Staphylococci* contains teichoic acid and peptidoglycan in which the diamino acid is L-lysine. Therefore, *Staphylococci* are susceptible to lysis by lysostaphin, but resistant to lysis by lysozyme. They are facultative anaerobes and metabolism respiratory and fermentative. The skin and the mucous membranes of humans and warm-blooded animals are the primary reservoirs for *Staphylococci*. They also can be found in air, dust, sewage, water, and food.

*Staphylococcus arlettae* is a new species in the genus *Staphylococcus* [35]. It is coagulase-negative and novobiocin-resistant. The cells are nonsporulating cocci 0.5-1.5 μm in diameter. It is mainly found on the skin and nares of poultry and goats. In this section, strain *Staphylococcus arlettae* (ATCC 43957) has been analyzed to provide a protein mass table for species *Staphylococcus arlettae*.

### 3.6.2 Experimental

#### 3.6.2.1 Chemicals and Materials

*Staphylococcus arlettae* (ATCC 43957) was grown in our lab from a stock of lyophilized cells (ATCC 43957) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding 200 μL of working stock culture into 50 mL of Trypticase soy broth. The culture was kept at 37 °C for 2 h. Then this starter culture was added into 1750 mL of scale-up culture. After 4 h, a portion of 300 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 4 h. This procedure was repeated, in the same manner, at 5, 12, 24, 48, 72, 144, and 288 h, respectively.

Trypticase soy broth was from Becton Dickinson (Becton Dickinson Biosciences, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

#### 3.6.2.2 Sample Preparation

For each of the 9 incubation times examined, *ca.* 10 mg of lyophilized cells were resuspened in 4 mL of MQ water. The cell suspension was passed through a Thermo Spectronic French pressure cell at 18,000 psi, and the pH of the suspension was adjusted to < 2 with 10 % TFA. After centrifuging, the precipitate was mixed with 1 mL of 50 mM Tris-HCl buffer (pH 8.5). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

#### 3.6.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.4.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:15, 30:35, 25:30, 50:45, 60:80, 70:2.

### 3.6.3 Results and Discussion

#### 3.6.3.1 Protein Extraction

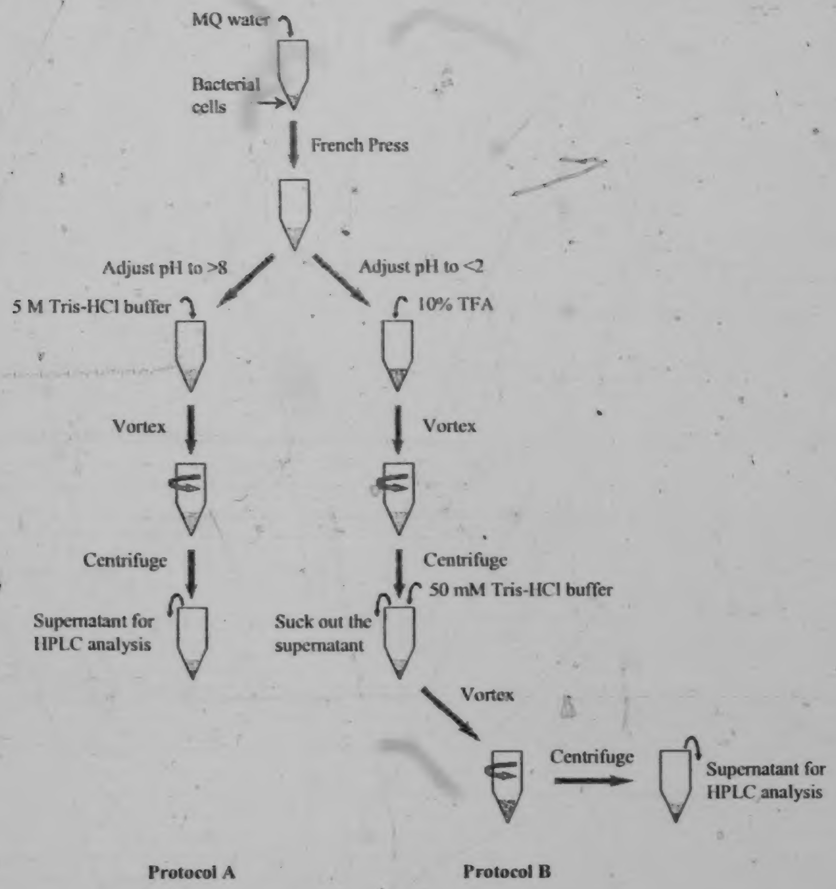
MQ water
Bacterial cells
French Press
Adjust pH to >8
Adjust pH to <2
5 M Tris-HCl buffer
10% TFA
Vortex
Vortex
Centrifuge
Centrifuge
50 mM Tris-HCl buffer
Supernatant for HPLC analysis
Suck out the supernatant
Vortex
Centrifuge
Supernatant for HPLC analysis
Protocol A
Protocol B

**Figure 3.7** Protein extraction protocols using French Press for Gram-positive bacteria.

Based on the previous work, we have established two protocols to extract proteins from Gram-positive cells with French Press (see Figure 3.7). For all the Gram-positive species described hereafter, both protocols were employed to determine the presence/absence of the "interference" components. For those containing of "interference" components, protocol B was used to remove the "interference". Otherwise, proteins are extracted using protocol A prior to HPLC/offline MALDI analysis. For *Staphylococcus arlettae* (ATCC 43957), protocol B was used due to the presence of the "interference" components (Figure 3.8).

### 3.6.3.2 Mass Tables

Protein extract from each sample harvested at various growth times was separated by using HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Staphylococcus arlettae* (ATCC 43957) (see Appendix, Table A.7) are same as those described in Section 3.1.3.2.

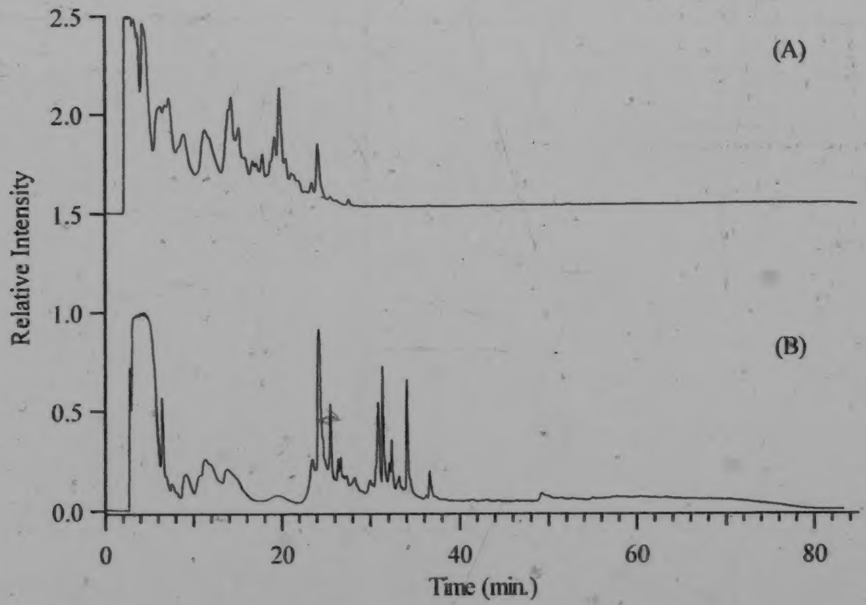

**Figure 3.8** Chromatograms of *Staphylococcus arlettae* (ATCC 43957) extracts using: (A) protocol A, (B) protocol B.

For this bacterium, there are 360 individual masses in total detected by LC/off-line MALDI over the entire incubation period. Table A.7 lists all of the $[M+H]^+$ values for each incubation time studied. As shown in Table A.7, more low masses (2 kDa–5 kDa) were observed in the samples than other bacteria we studied. They were mainly detected in the samples harvested from 5 to 72 h. The sample collected at 24 h possessed the largest number of low mass entries among all the samples, while only 8 low mass entries were observed in the sample harvested from 288 h. High masses (6 kDa–10 kDa) were mainly detected in the samples collected from longer growth time (72 h–288 h). And most 10 kDa masses arise from the 288 h sample. There are only 19 mass entries observed in the sample collected from lag phase (4 h). With external calibration, the mass measurement accuracy is expected to be better than 0.05%.

## 3.7 *Staphylococcus kloosii*

### 3.7.1 Introduction

The species *Staphylococcus kloosii* is a new member of the genus *Staphylococcus* [35]. It is coagulase-negative and novobiocin-resistant. The cells are nonsporulating cocci 0.6-1.8 μm in diameter. It is isolated mainly from the skin of various wild animals and rarely on that of farm animals. In this section, strain *Staphylococcus kloosii* (ATCC 43959) has been analyzed to provide a protein mass table for species *Staphylococcus kloosii.*

### 3.7.2 Experimental

#### 3.7.2.1 Chemicals and Materials

*Staphylococcus kloosii* (ATCC 43959) was grown in our lab from a stock of lyophilized cells (ATCC 43959) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding a loop of working stock into 20 mL of Trypticase soy broth. The culture was kept at 37 °C for approx. 24 h. Then appropriate volume of starter culture was added into 500 mL of scale-up culture. After 3 h, a portion of 100 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water. Some cells were resuspended in cold MQ water for downstream analysis. Other cells were lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 3 h. The same procedure was repeated at 4, 6, 12, 24, 48, 72, 144, 192 h, 292 h, and 484 h, respectively.

Trypticase soy broth was from (Becton Dickinson Biosciences, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

### 3.7.2.2 Sample Preparation

For each of the 11 incubation times examined, fresh cells were resuspended in 4 mL of cold MQ water. The cell suspension was passed through a Thermo Spectronic French pressure cell at 18,000 psi and low temperature. Then the pH of the suspension was adjusted to < 2 with 10 % TFA. After centrifuging, the precipitate was mixed with 2 mL of 50 mM Tris-HCl buffer (pH 8.5). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 3.7.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.4.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:15, 40:40, 60:80, and 70:2.

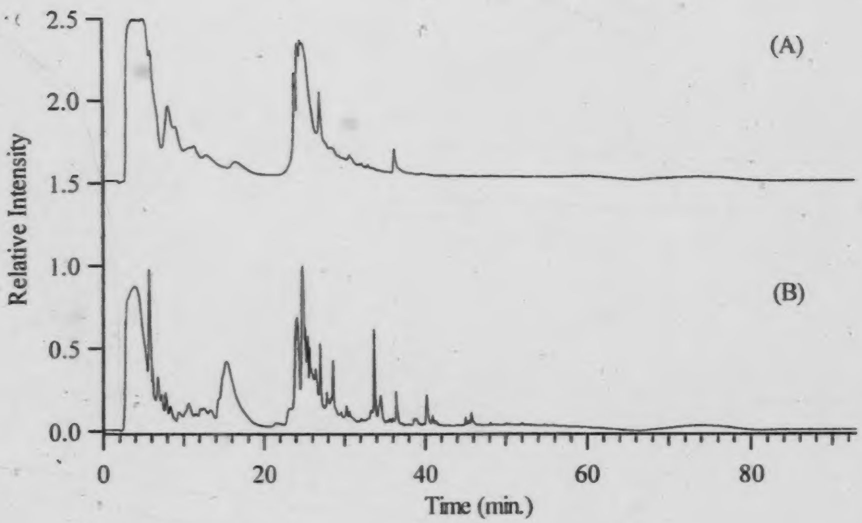

**Figure 3.9** Chromatograms of *Staphylococcus kloosii* (ATCC 43959) extracts using (A) protocol A, (B) protocol B.

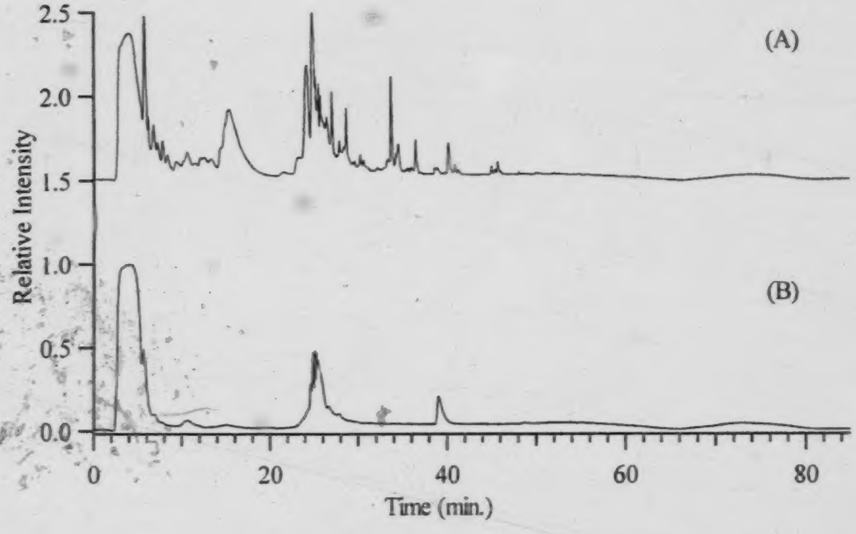

**Figure 3.10** Comparision of chromatograms: (A) Chromatogram of the *Staphylococcus kloosii* (ATCC 43959) extract from fresh cells harvested at 48 h. (B) Chromatogram of the *Staphylococcus kloosii* (ATCC 43959) extract from lyophilized cells harvested at 48 h.

### 3.7.3 Results and Discussion

#### 3.7.3.1 Protein Extraction

French Press was used to extract proteins from *Staphylococcus kloosii* (ATCC 43959) since it is Gram-positive. From Figure 3.9, we can see that there are “interference” components in the protein extract from *Staphylococcus kloosii* (ATCC 43959) cells. So, proteins from *Staphylococcus kloosii* (ATCC 43959) cells were extracted using protocol B.

There was obvious difference between protein extract from *Staphylococcus kloosii* (ATCC 43959) fresh cells and that from lyophilized cells. In Figure 3.10, trace A

**Table 3.3** $[M+H]^+$ (Da) from fresh *Staphylococcus kloosii* (ATCC 43959) cells harvested at 48 h by LC/off-line MALDI.

| | | | | | |
|---|---|---|---|---|---|
| 2030 | 2512 | 3879 | 5046 | 6585 | 9672 |
| 2068 | 2530 | 4596 | 5095 | 6642 | 11589 |
| 2086 | 2612 | 4713 | 5227 | 6944 | |
| 2185 | 2846 | 4864 | 5602 | 7037 | |
| 2465 | 3129 | 4990 | 6511 | 9027 | |

**Table 3.4** $[M+H]^+$ (Da) from lyophilized *Staphylococcus kloosii* (ATCC 43959) cells harvested at 48 h by LC/off-line MALDI.

| | | | | | |
|---|---|---|---|---|---|
| 2011 | 2712 | 4375 | 5121 | 6511 | 7482 |
| 2025 | 2787 | 4456 | 5173 | 6566 | 7512 |
| 2069 | 2799 | 4595 | 5226 | 6584 | 8032 |
| 2105 | 3082 | 4670 | 5434 | 6642 | 8976 |
| 2110 | 3128 | 4862 | 5602 | 6693 | 9026 |
| 2168 | 3220 | 4945 | 5993 | 6869 | 9672 |
| 2173 | 3237 | 4989 | 6117 | 6943 | 9741 |
| 2197 | 3247 | 5048 | 6353 | 6995 | 9764 |
| 2324 | 3403 | 5072 | 6369 | 7036 | 9895 |
| 2391 | 3676 | 5085 | 6396 | 7167 | 10019 |
| 2454 | 3747 | 5090 | 6455 | 7350 | 10151 |
| 2546 | 4174 | 5094 | 6488 | 7379 | 11590 |

shows the chromatogram of the protein extract from the fresh cells harvested at 48 h, while the chromatogram of protein extract from lyophilized cells collected at 48 h is shown in trace B. From Figure 3.10, it is obvious that much more proteins can be detected in the extract from the fresh cells than the lyophilized sample. To investigate the difference between these two samples, both of them were tested by LC/off-line MALDI MS, and the results are listed in Table 3.3 and 3.4. There are only 27 mass entries observed in the lyophilized sample and 72 mass entries are detected from the fresh cells.

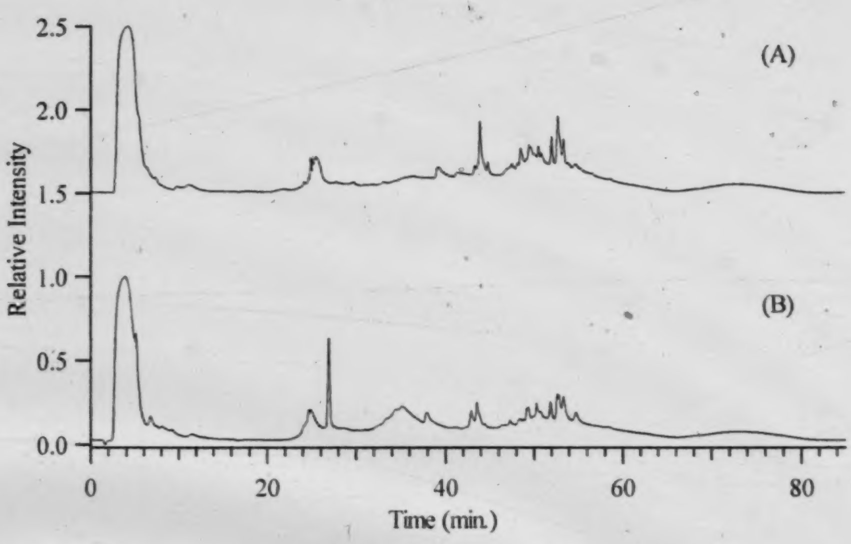

**Figure 3.11** Comparision of chromatograms: (A) Chromatogram of the fresh S. *kloosii* (ATCC 43959) extract from fresh cells harvested at 192 h. (B) Chromatogram of the same extract that was stored for 2 days.

*Staphylococcus kloosii* (ATCC 43959) protein extract shows such a poor stability that they can last for only a few days. The longer the sample is stored, the more the proteins are lost since more precipitates are formed during the acidification of the Tris extract. In Figure 3.11, trace A shows the chromatograph of fresh protein extract from fresh cells collected at 192 h, while trace B shows the chromatograph of protein extract that was stored for 2 days (from fresh cells collected at 192 h). All experimental conditions were kept same except the origins of the samples were different. More peaks with short retention time (20-40 min.) appear in trace B (Figure 3.11), indicating possible degradation of some high molecular weight proteins during the storage period. The overall peak intensity in trace B are also lower so that some proteins might not be detectable in the MALDI experiment.

Based on these comparisons, in our experiments, extracted proteins from fresh cells were immediately analyzed by HPLC/offline MALDI so as to minimize the protein loss.

### 3.7.3.2 Mass Tables

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Staphylococcus kloosii* (ATCC 43959) (see Appendix, Table A.8) are same as those described in Section 3.1.3.2. For this bacterium, there are 294 individual masses in total detected by LC/off-line MALDI over the entire incubation period. Table A.8 lists all of the $[M+H]^+$ values for each incubation time studied. With external calibration, the mass measurement accuracy is expected to be better than 0.05%.

As shown in Table A.8, more low masses (2 kDa–4 kDa) are observed in the samples harvested in shorter growth time (3–72 h) than those collected in longer period (144–484 h) when only 8 mass entries were detected. Interestingly, high masses (10 kDa–30 kDa) were mainly detected in the samples both harvested in longer period (144–484 h) and collected at 3 h. In the low mass range (2 kDa–4 kDa), mass data from the sample obtained at 3 h was similar to that of shorter growth time (3–72 h). However, in the high mass region (8 kDa–20 kDa), the results of 3, 144, 192, 292, and 484 h samples were comparable. This presents an interesting pattern that the proteins detected from cells at the longer growth period show similarity to those from earlier growth period. The sample collected at 3 h possessed the largest number of mass entries among all the samples in this experiment.

## 3.8 *Streptococcus bovis*

### 3.8.1 Introduction

The genus *Streptococcus* is in the family of *Lactobacillaceae*. Cells are normally spherical or elongated with less than 2 µm in diameter. They appear as chains under microscopic observation due to dividing in one plane only. *Streptococci* are Gram-positive bacteria. "*Bergey's Manual of Determinative Bacteriology*" describes it as:

> *Endospores are not formed. Most are facultatively anaerobic. Chemooranotrophs, with fermentative metabolism. Catalase-negative. Nutritional requirements are complex and variable. Many species exist as commensals or parasites on man or animal, some are highly pathogenic* [36].

*Streptococcus bovis* is a bacterium that is normally found in the rumen of ruminant animals such as cattle and sheep and has potential as an industrial organism for production of such products as lactate and ethanol. Cells are 0.8-1.0 µm in diameter in pairs and moderate to long chains. This species is rarely isolated from humans but has been reported to cause diseases such as endocarditis and meningitis. In this section, strain *Streptococcus bovis* (ATCC 33317) has been analyzed to provide a protein mass table for species *Streptococcus bovis*.

### 3.8.2 Experimental

#### 3.8.2.1 Chemicals and Materials

*Streptococcus bovis* (ATCC 33317) was grown in our lab from a stock of lyophilized cells (ATCC 33317) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding a loop of working stock into 50 mL of Brain heart infusion broth. The culture was kept at 37 °C overnight. An appropriate volume of starter culture was added into 1650 mL of scale-up culture. After 3 h, a portion of 150 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 3 h. This procedure was repeated, in the same manner, at 5, 12, 24, 48, 96, 168, and 336 h, respectively.

Brain heart infusion broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

#### 3.8.2.2 Sample Preparation

For each of the 8 incubation times examined, *ca.* 10 mg of lyophilized cells were resuspened in 4 mL of MQ water. The cell suspension was passed through the Thermo Spectronic French pressure cell at 18,000 psi. The pH value of the suspension was then adjusted to 8.5 with 5 M Tris buffer. After the suspension was centrifuged at 11,750 *g*, the supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 3.8.2.3 Total Protein Determination

The Tris extracts were mixed with Coomassie plus protein assay reagent and the absorbance was read at 590 nm. A standard curve was prepared using bovine gamma globulin (Pierce).

### 3.8.2.4 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.4.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:25, 60:50, 70:80, and 80:2.

### 3.8.3 Results and Discussion

*Streptococcus bovis* (ATCC 33317) is also a Gram-positive bacterium; therefore, French Press was used to extract the proteins from the cells. Protocol A was used to due to absence of the "interference" components (Figure 3.12).

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Streptococcus bovis* (ATCC 33317) (see Appendix, Table A.9) are same as those described in Section 3.1.3.2.

For this bacterium, there are 512 individual masses in total detected by LC/off-line MALDI over the entire course of incubation. Table A.9 lists all of the $[M+H]^+$ values for each incubation time studied. With external calibration, the mass measurement

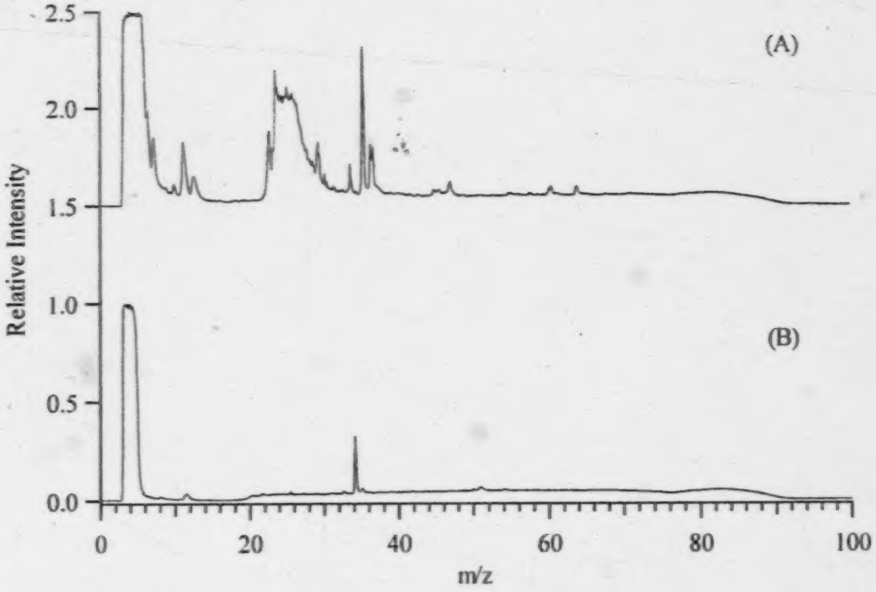

**Figure 3.12** Chromatograms of *Streptococcus bovis* (ATCC 33317) extracts using (A) protocol A, (B) protocol B.

accuracy is expected to be better than 0.05%. As shown in Table A.9, the sample harvested at 48 h differs from the samples collected at other growth times. It possesses the minimal mass entry number among all the samples in this experiment; however, the protein concentration in its extract is the highest (10.111 μg/μL). Moreover, 2 kDa mass entries were not observed in the sample obtained at 48 h. High mass entries (10 kDa-33kDa) were mainly detected at the samples both harvested at the shorter growth time (3 h) and longer growth time (48-336 h), while higher mass entries were only observed in the samples collected after 48 h.

### 3.9 *Microbacterium saperdae*

#### 3.9.1 Introduction

The genus *Microbacterium* is in the family of *Corynebacteriaceae*. It is small and is irregular rods (0.4-0.8 µm in diameter by 1.0-4.0 µm in length) in young cultures (12-24 h). While in older cultures (3-7 days), the cells become shorter [37]. Some of them may be coccid. However, the rodcoccus growth cycle does not happen. This genus is Gram-positive and non-sporeforming bacterium. It is not motile or motile by one to three flagella. The cell growth is aerobic. Mariko Takeuchi and Kazunuri Hatano suggested the genera *Aureobactreium* and *Microbacterium* be unified [38]. An amended genus *Microbacterium* was proposed for the two combined genera.

The species *Microbacterium saperdae* (Lysenko) (note that Hatano deposited this strain as *Brevibacterium saperdae* Lysenko [37]) is straight or slightly curved rods with round ends (0.4-0.8 µm in diameter by 0.8-2.0 µm in length). It is Gram-variable in young culture while it is Gram-positive in older culture. This species is nonmotile, non-sporeforming and no capsules. In this section, strain *Microbacterium Saperdae* (ATCC 19272) has been analyzed to provide a protein mass table for species *Microbacterium Saperdae*.

### 3.9.2 Experimental

#### 3.9.2.1 Chemicals and Materials

*Microbacterium Saperdae* (ATCC 19272) was grown in our lab from a stock of lyophilized cells (ATCC 19272) purchased from American Type Culture Collection (ATCC). The starter culture was prepared by adding a loop of working stock into 50 mL of nutrient broth. The culture was kept at 30 °C for 2 h. Then this starter culture was added into 1700 mL of scale-up culture. After 18 h, a portion of 300 mL was transferred to a centrifuge tube and spun for 20 min. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 18 h. This procedure was repeated, in the same manner, at 20, 24, 32, 48, 72, 144, and 360 h, respectively.

Nutrient broth was from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile was from Fisher Scientific Canada (Edmonton, AB).

#### 3.9.2.2 Sample Preparation

For each of the 8 incubation times examined, *ca.* 12 mg of lyophilized cells were resuspened in 4 mL of MQ water. The cell suspension was passed through the Thermo Spectronic French pressure cell at 18,000 psi. The pH value of the suspension was then adjusted to 8.5 with 5 M Tris buffer. After the suspension was centrifuged at 11,750 *g*, the supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

#### 3.9.2.3 Mass Spectrometry

**HPLC/off-line MALDI MS.** HPLC/off-line MALDI MS experiment was performed as described in Section 3.4.2.3. However, in the HPLC experiment, the gradient elution was employed using the following times and % acetonitrile (min: %ACN) 0:2, 15:25, 55:50, 65:80, and 70:2.

### 3.9.3 Results and Discussion

*Microbacterium saperdae* is a Gram-positive bacterium; therefore, French Press was used to extract proteins from *Microbacterium saperdae* (ATCC 19272) cells. Protocol A was used to due to absence of the "interference" components (Figure 3.13).

Protein extract from each sample harvested at various growth times was separated by HPLC. Each HPLC fraction was detected by MALDI. The selection and exclusion of mass peaks and the creation of mass table for *Microbacterium saperdae* (ATCC 19272) (see Appendix, Table A.10) are same as those described in Section 3.1.3.2.

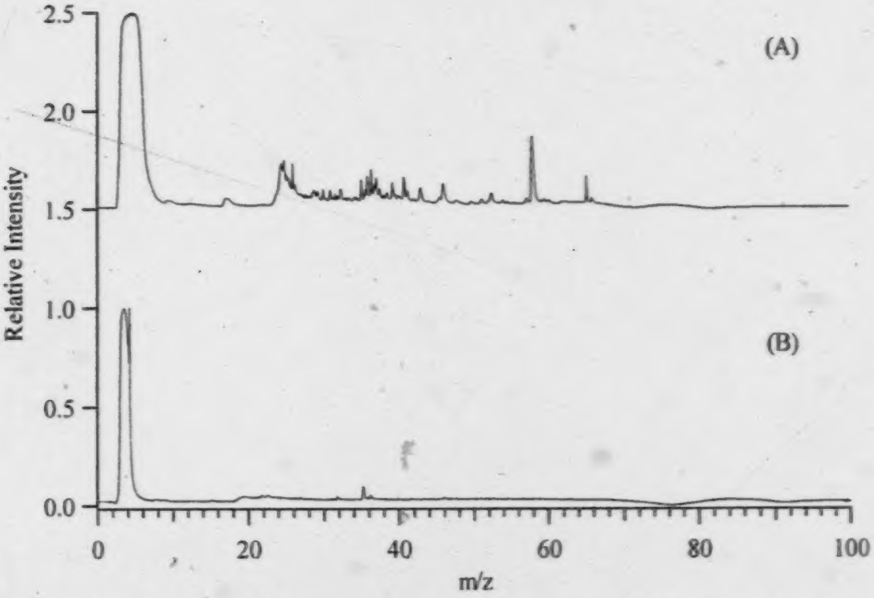

**Figure 3.13** Chromatograms of *Microbacterium saperdae* (ATCC 19272) extracts using (A) protocol A, (B) protocol B.

For this bacterium, there are 279 individual masses in total detected by LC/off-line MALDI over the entire incubation period. Table A.10 lists all of the $[M+H]^+$ values for each incubation time studied. As shown in Table A.10, less protein entries were observed in shorter growth time (18 h and 20 h), while more protein entries were detected in longer period (24–360 h). However, low masses (2 kDa–3 kDa) were mainly observed in the samples harvested from 24 to 360 h. Interestingly, high masses (10 kDa–20 kDa) were detected in samples from shorter growth time (18 h and 24 h), and high mass over 18 kDa was not observed in all samples. With external calibration, the mass measurement accuracy is expected to be better than 0.05%.

### 3.10 Conclusions

An experimentally derived bacterial protein mass database that contains nine bacterial species, namely, *E. coli*, *Bacillus globigii*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus arlettae*, *Staphylococcus kloosii*, *Streptococcus bovis*, *Microbacterium saperdae*, and *Pseudomonas fluorescens* has been established. For each species, mass data (typically at the mass range from 2-20 kDa) obtained from various growth periods were compiled to give one protein mass table, and the set of these tables from nine different known bacterial species comprised the current experimentally derived protein mass database. In this way, the microbiological factor of varying growth times is coherently included in the database. Moreover, database created in this method can reflect certain information of post-translational modification. Finally, on the basis of theoretical analysis and our further experimental tests, this experimentally derived protein mass database has proven accurate and reliable in the identification/discrimination of bacteria (Chapter 5).

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# Chapter 4

# Expression Analysis of Low Mass Bacterial proteome by Using Mass Spectrometry

## 4.1 Introduction

Although numerous reports on bacterial identification using various biomarkers have been published in recent literatures [1-23], there have been few successes among them due to poor spectral reproducibility of the complex protein extracts [4, 17, 18, 21]. There are two main factors impeding the protein profiling. One is experimental factor such as protein extraction solvent, protein extraction method, and sample/matrix solvent, *etc.* Those experimental conditions, however, can be controlled by considerable effort on using the same optimum protocol, such that reproducible mass spectra can be obtained [4]. Another factor is microbiological factor, e.g., bacteria growth times and growth cultures [17, 24]. Bacteria usually respond rapidly and drastically to environmental changes, thus resulting in the variation of the protein profiling. In bacterial identification, unknown samples usually come from different environments and the growth conditions are mostly out of control. Therefore, understanding of protein expression as a function of cell culture is a key to solve the irreproducibility problem in MS-based bacterial identification.

Three approaches utilizing mass spectrometry have been developed for bacterial protein profiling. The most commonly used method of monitoring cellular protein expression is two-dimensional polyacrylamide gel electrophoresis (2D PAGE) [25-40]. In this method, protein separations are based on the isoelectric points and molecular weight of proteins. The 2D spot pattern provides an indicator of changes in protein expression. The further protein identification following 2D-PAGE involves the in-gel digestion and analysis using mass spectrometry [33, 41, 42]. The limitations associated with 2D-PAGE are low resolution, intensive labour, slow analysis and poor reproducibility. Moreover, a single protein always leads to several different spots due to post-translational modification [43,44]. An alternative to 2D-PAGE involves combining liquid chromatograph with mass spectrometry, such as HPLC/off-line MALDI and HPLC/ESI-MS [45-60]. In this method, proteins are separated quickly via the column. The mass spectrum of each fraction is then recorded by a down-stream mass spectrometer, which directly provides the protein profiles. The newest approach for the profiling proteins involves digestion of a protein extract, followed by separation of the peptides using multidimensional chromatography and peptide sequencing using tandem mass spectrometry [61-70]. Identification of one or more peptides from a protein demonstrates the existence of that protein, therefore, giving the protein profiles.

In this chapter, we report a study focusing on the low mass protein expression of bacterial cells as a function of cell culture. *E. coli* (ATCC 9637), *E. coli* (ATCC 47076), *Bacillus globigii* (ATCC 9372), *Bacillus subtilis* (ATCC 23857), *Bacillus megaterium* (ATCC 14581), *Staphylococcus arlettae* (ATCC 43957), *Staphylococcus kloosii* (ATCC 43959), *Streptococcus bovis* (ATCC 33317), *Microbacterium saperdae* (ATCC 19272), and *Pseudomonas fluorescens* (ATCC 948) were chosen as model bacteria for investigation. Protein expression profiles were obtained by LC/ESI-MS and LC/off-line MS, as they can provide protein expression directly and rapidly.

## 4.2 Experimental

*E. coli* (ATCC 9637) was grown in two batches. In the first batch, it was grown from a stock of lyophilized cells (ATCC 9637) purchased from Sigma. The 48 h lyophilized sample from the first batch was subsequently used as the starter for cell growth of the second batch. Experimental details for the first batch are reported in Chapter 2. The experimental of the second batch is described as following.

For the experimental of *E. coli* (ATCC 47076), *Bacillus globigii* (ATCC 9372), *Bacillus subtilis* (ATCC 23857), *Bacillus megaterium* (ATCC 14581), *Staphylococcus arlettae* (ATCC 43957), *Staphylococcus kloosii* (ATCC 43959), *Streptococcus bovis* (ATCC 33317), *Microbacterium saperdae* (ATCC 19272), and *Pseudomonas fluorescens* (ATCC 948) see Chapter 3.

### 4.2.1 Chemicals and Materials

*E. coli* (ATCC 9637) was grown in Luria Broth (LB) (BBL, Becton Dickinson) at 37 °C with constant shaking. The starter culture was prepared by adding 40 mg of 48 h lyophilized sample from previous batch into 50 mL of culture. The culture was kept at 37 °C for 2 h. Then this starter culture was added into 1550ml of scale-up culture. After 2 h, a portion of 100 mL was transferred to a centrifuge tube and spun for 20 minutes. The supernatant was then removed and the cells were rinsed with MQ water, lyophilized and stored at –25 °C until analysis. This sample is thereof specified as a cell incubation time of 2 h. The same procedure was repeated at 3, 4, 6, 8, 12, 16, 20, 24, 36, 48, and 72 h, respectively.

Luria Broth was from Becton Dickinson Biosciences (Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich-

Fluka (Oakville, Ontario, Canada). HPLC grade acetonitrile and glacial acetic were from Fisher Scientific Canada (Edmonton, AB).

### 4.2.2 Sample Preparation

For each of the 12 incubation times examined, *ca.* 11 mg of lyophilized cells was suspended in 2 mL of 0.1% (v/v) TFA. The mixture was cooled with an ice-water bath and sonicated with a Branson probe sonicator. The probe tip of the sonication unit was immersed in the solution and the sonication was conducted for 1 min (pulsed mode: 1 pulse per second, pulse duration: 0.75 seconds). The mixture was centrifuged at 11,750 *g*. The supernatant was filtered using Mirocon-3 filters with 3,000 Da molecular mass cutoff (Millipore).

### 4.2.3 Mass Spectrometry

**LC/ESI-quadrupole.** Solvent delivery and separations were performed on an Agilent (Palo Alto, CA) HP1100 HPLC system. Chromatographic analysis was performed with a reversed-phase Vydac $C_8$ column (300Å, 5μm, 2.1mm i.d. × 150mm). The mobile phase consisted of $H_2O$ and acetonitrile, both with 0.05% (v/v) TFA. Gradient elution at 200 μL/min was applied using the following times and % acetonitrile (min:%ACN) 0:2, 10:20, 40:40, 45:55, 60:90, 65:2. Post-column glacial acetic acid was infused at 100 μL/min by a polyetheretherkrtone (PEEK) "Y" connector and syringe pump (Cole Parmer, Vernon Hills, IL). The HPLC effluent was analyzed with an Agilent 1100 series MSD quadrupole mass spectrometer. A variable fragmentation voltage was used during the ESI detection. The optimized voltage ramp was found to be at *m/z* 500, 60 V; at *m/z* 1000, 120 V; at *m/z* 3000, 220 V.

## 4.3 Results and Discussion

### 4.3.1 Protein Expression changes as a Function of Cell Growth Time.

Tables 4.1-4.9 list the percentages of the number of mass entries detected in each sample harvested at different growth time for nine bacteria listed in our experimentally derived bacterial protein mass database. Those tables indicate that the overlay of masses obtained in different growth time samples is actually insignificant. The samples collected at different growth time posses their own characteristic masses. These observations suggest that protein expression is quite sensitive to growth time. Since, for each bacterium, all the samples were harvested according to the growth curve, protein expression is sensitive to the growth phase in a cell growth curve in the strict sense.

Protein mass tables for nine bacterial species containing the mass values of protonated molecular ions from samples harvested at different growth times were listed in Tables A.2-A.10 (see Appendix). It is noteworthy that some masses are observed in a few growth times, some masses are observed in every growth time, and some masses are only observed in one growth time. The masses detected over the entire growth period always posses high intensity in both UV traces and MALDI spectra, indicating that the proteins with those masses might have high quantity in cells and are easily ionized. Therefore, they are characteristic masses of the corresponding bacterium and play an important role in bacterial identification. The masses observed in one growth time are characteristic masses of that very period. They can provide growth time information in bacterial identification. For those masses observed in a number of growth times, it is very interesting that some of them were detected in several continuous growth times; while others were observed in several disconnected points.

**Table 4.1** The percentage of the number of mass entries detected in each *E. coli* (ATCC 47076) extract from different growth time.

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| 17% | 32% | 32% | 27% | 38% | 29% |

**Table 4.2** The percentage of the number of mass entries detected in each *Pseudomonas fluorescens* (ATCC 948) extract from different growth time.

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
| 24% | 16% | 34% | 37% | 38% | 37% | 28% | 33% | 27% |

**Table 4.3** The percentage of the number of mass entries detected in each *Bacillus globigii* (ATCC 9372) extract from different growth time.

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| 27% | 30% | 25% | 27% | 25% | 19% |

**Table 4.4** The percentage of the number of mass entries detected in each *Bacillus subtilis* (ATCC 23857) extract from different growth time.

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| 24% | 19% | 25% | 16% | 20% | 24% | 31% | 32% |

**Table 4.5** The percentage of the number of mass entries detected in each *Bacillus megaterium* (ATCC 14581) extract from different growth time.

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 15% | 28% | 34% | 31% | 25% | 22% | 24% | 28% |

**Table 4.6** The percentage of the number of mass entries detected in each *Staphylococcus arlettae* (ATCC 43957) extract from different growth time.

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| 5% | 19% | 26% | 41% | 36% | 31% | 14% | 19% |

**Table 4.7** The percentage of the number of mass entries detected in each *Staphylococcus kloosii* (ATCC 43959) extract from different growth time.

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| 32% | 10% | 12% | 20% | 16% | 23% | 21% | 25% | 26% | 23% | 18% |

**Table 4.8** The percentage of the number of mass entries detected in each *Streptococcus bovis* (ATCC 33317) extract from different growth time.

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 28% | 21% | 19% | 22% | 21% | 39% | 31% | 27% |

**Table 4.9** The percentage of the number of mass entries detected in each *Microbacterium saperdae* (ATCC 19272) extract from different growth time.

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| 13% | 17% | 25% | 21% | 23% | 25% | 33% | 24% |

Proteins with characteristic mass observed in several continuous growth periods exist in the bacterial cells for a certain period of time which may be longer than that observed in the database table. They always possess relative high signal to noise ratio (S/N) in MALDI spectra. The following examples will illustrate this point in detail.

The mass 8406 Da was continuously observed in the *Pseudomonas fluorescens* (ATCC 948) extracts from the samples harvested from 20 h to 432 h other than 18 h (see Figure 4.1). It started to present in the *Pseudomonas fluorescens* (ATCC 948) cells from growth time of 20 h. With the growth of the cells, the intensity of the peak keeps increasing till 120 h when this protein becomes the dominant protein in the MALDI spectrum of the corresponding HPLC fraction. Afterwards the intensity of the peak amount starts to decrease. Finally, the peak at m/z 8406 is difficult to be observed in the corresponding HPLC fraction of extract from the 432 h sample since the intensity of the peak at mass 8406 Da becomes considerably low. From Figure 4.1, it is very clear that protein with characteristic mass 8406 Da exist in the cell extract for a certain period of time (20-432h). However, the mass 8406 Da detected from the 20 and 432 h samples is not listed in the database table because the S/N ratio of peak at m/z 8406 Da observed in two samples is less than 3 (see Appendix, Table A.3).

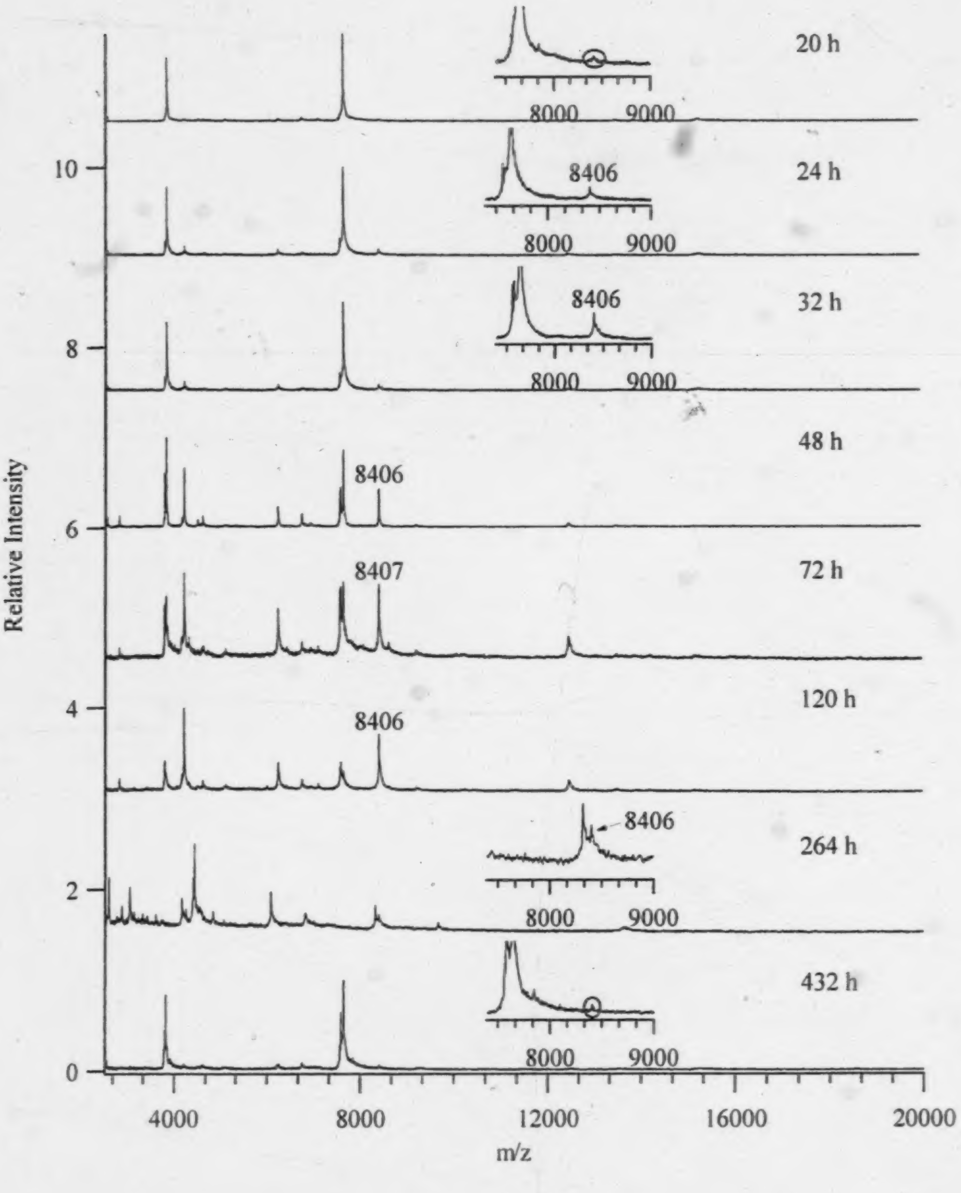

**Figure 4.1** MALDI spectra of corresponding HPLC fractions consisting of mass 8406 Da of *Pseudomonas fluorescens* (ATCC 948) extract.

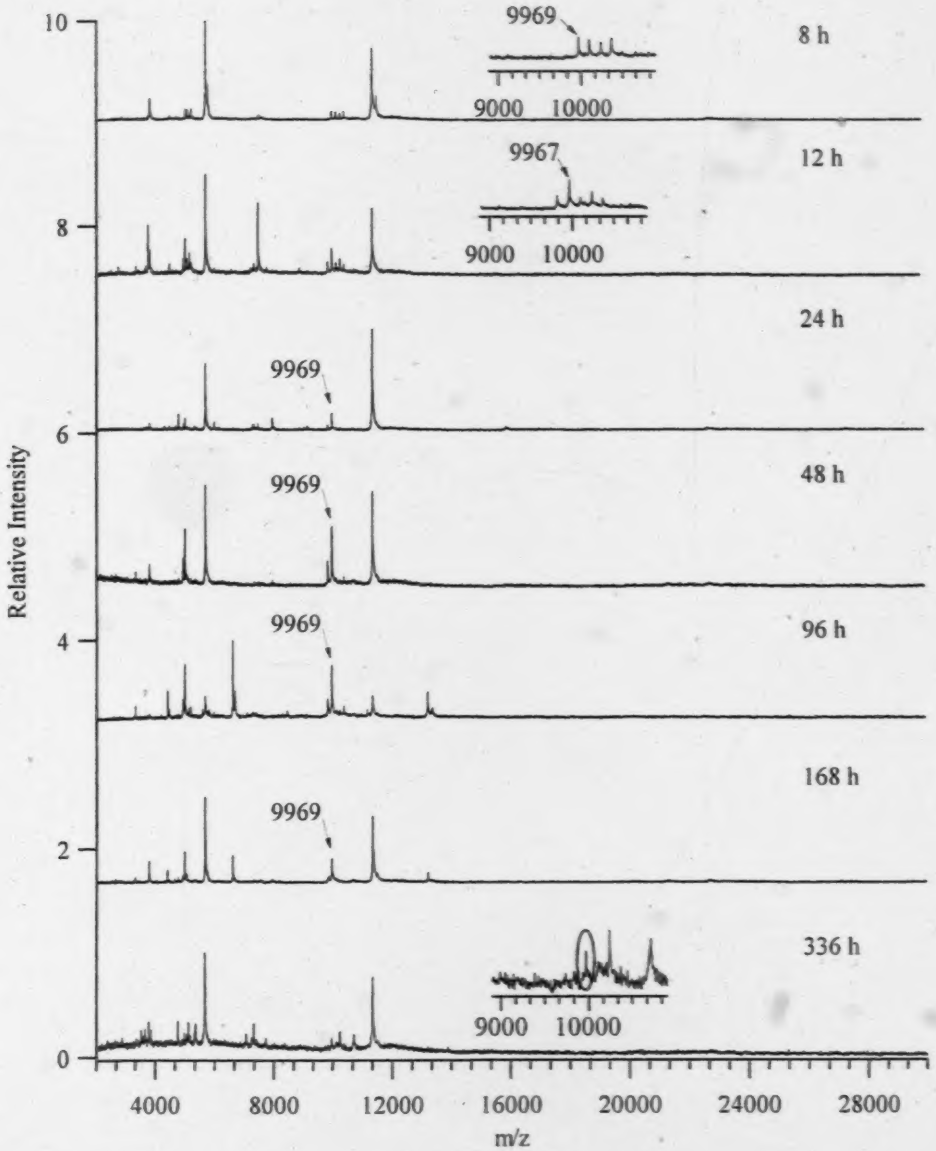

**Figure 4.2** MALDI spectra of corresponding HPLC fractions consisting of mass 9969 Da of *Bacillus megaterium* (ATCC 14581) extract.

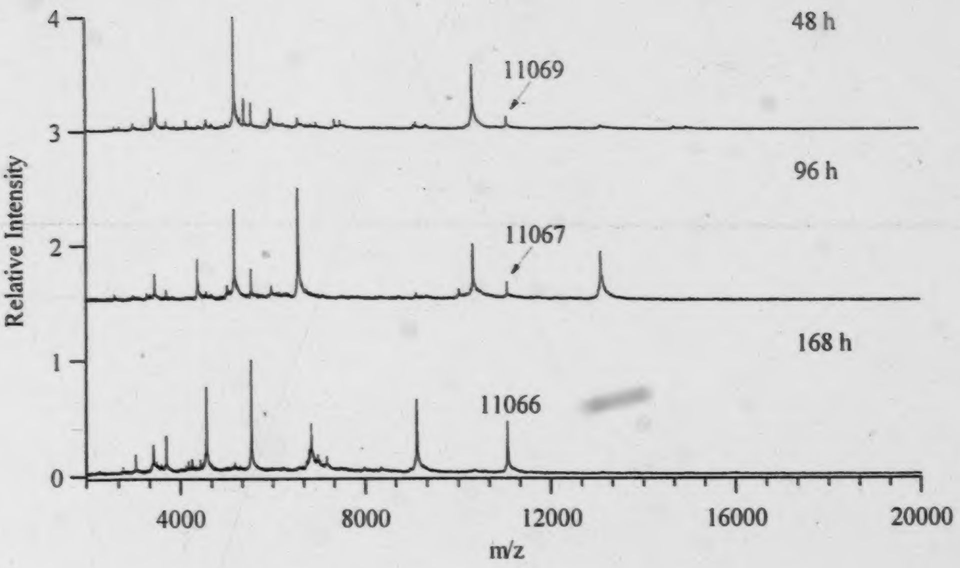

**Figure 4.3** MALDI spectra of corresponding HPLC fractions consisting of mass 11067 Da of *Streptococcus bovis* (ATCC 33317) extract.

Likewise, in Table A.6, the mass 9969 Da was observed in the *Bacillus megaterium* (ATCC 14581) cell extracts from the 8 to 168 h samples. From Figure 4.2, it can be seen that the peak at m/z 9969 Da was not observed in the 4 h sample; it actually exist in the cell extract in the growth period between 8 and 336 h. In the MALDI spectrum of corresponding HPLC fractions of the 336 h sample, the S/N ratio of peak at m/z 9969 Da is too low to observed. Therefore, the mass 9969 Da detected from 336 h is not listed in the database table for species *Bacillus megaterium*.

The mass 11067 Da is detected in the *Streptococcus bovis* (ATCC 33317) cell extracts from the 48 to 168 h samples (see Figure 4.3) as observed in the database table (see Appendix, Table A.9). Protein with characteristic mass 11067 Da only presents in the *Streptococcus bovis* (ATCC 33317) cells in quite a short time scale.

Proteins with characteristic masses observed in several disconnected growth time always possess low S/N in MALDI spectra. In fact, they also exist in the bacterial cells for a particular period. However, they are not observed in some growth time samples due to the experimental factors. In Table A.3 (see Appendix), the mass 14909 Da (average of the masses listed in the database table) can be observed in 18-24h, 48-120h and 432 h samples. In fact, it can de detected in the *Pseudomonas fluorescens* (ATCC 948) extracts from the 18 h to 432 h except 264 h samples (see Figure 4.4). For the 32 h sample, the S/N ratio of peak at m/z 14909 is less than 3 due to the poor quality of this spectrum. It is not listed in the database table. The reason for such a poor quality is likely because of the protein loss during the transfer process. Peak at *m/z* 14909 was not detected in the 264 h sample. The reason for that is still unclear. In the database table for species *Bacillus megaterium* (see Table A.6), the mass 6852 Da can be found in the extracts from the samples harvested at 8, 12, and 336 h. However, from Figure 4.5, we can see that the peak at *m/z* 6852 Da can be observed from the 8 to 336 h samples. In the spectra from the 24-168 h samples, the S/N ratio of peak at m/z 6852 Da is less than 3 due to suppression from the peaks at m/z 7376, 7344, and 7416 Da. Therefore, the mass 6852 Da detected from the 24-168 h samples is not listed in the database table for species *Bacillus megaterium*. Similarly, the mass 7879 Da can be detected in the *Streptococcus bovis* (ATCC 33317) cell extracts from the samples collected from 5 to 24 h. However, the peak at m/z 7879 Da observed in the 12 h sample was not listed in the database table since its S/N ratio is too low (less than 3). Some factors may account for this result.

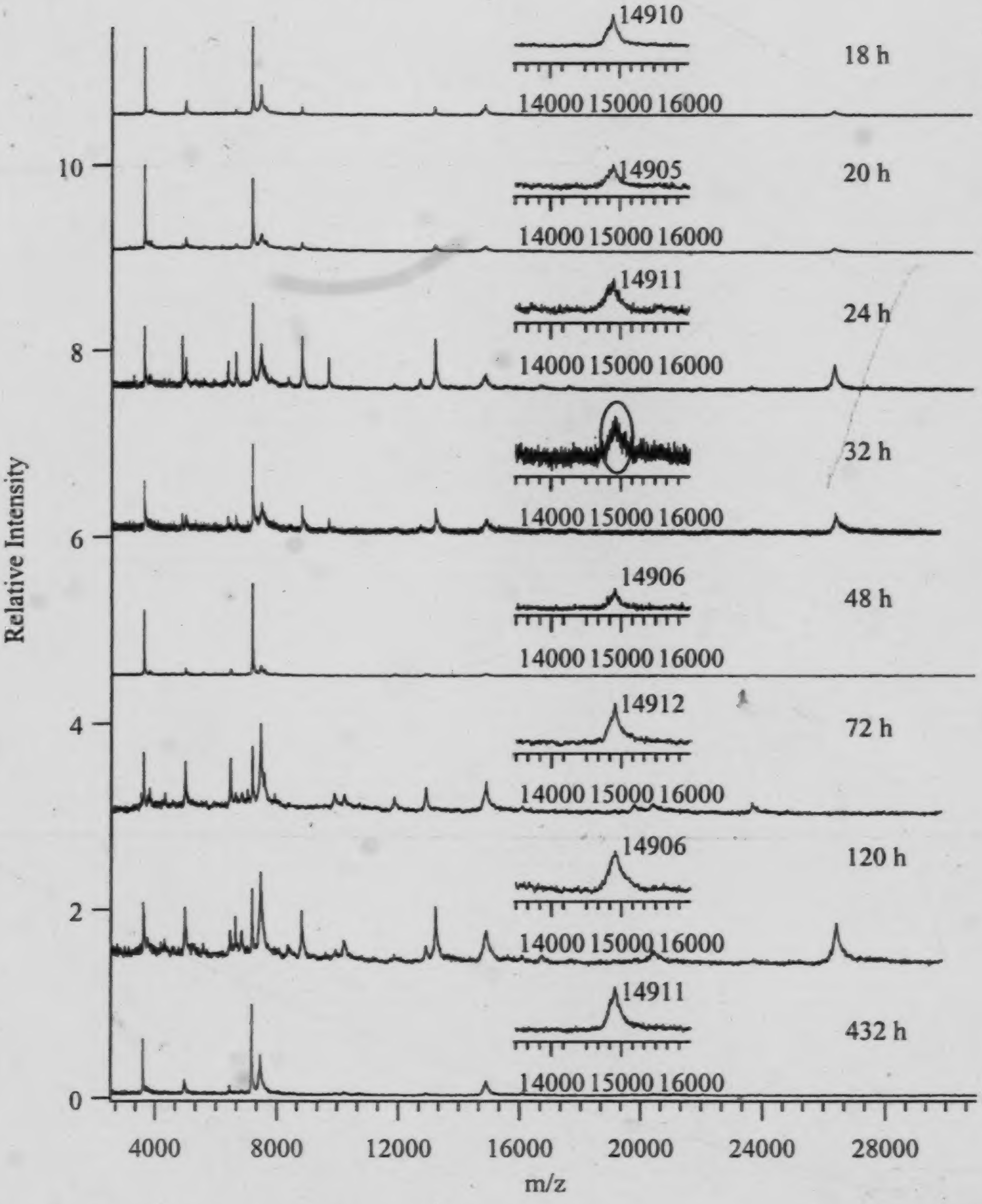

**Figure 4.4** MALDI spectra of corresponding HPLC fractions consisting of mass 14909 Da of *Pseudomonas fluorescens* (ATCC 948) extract.

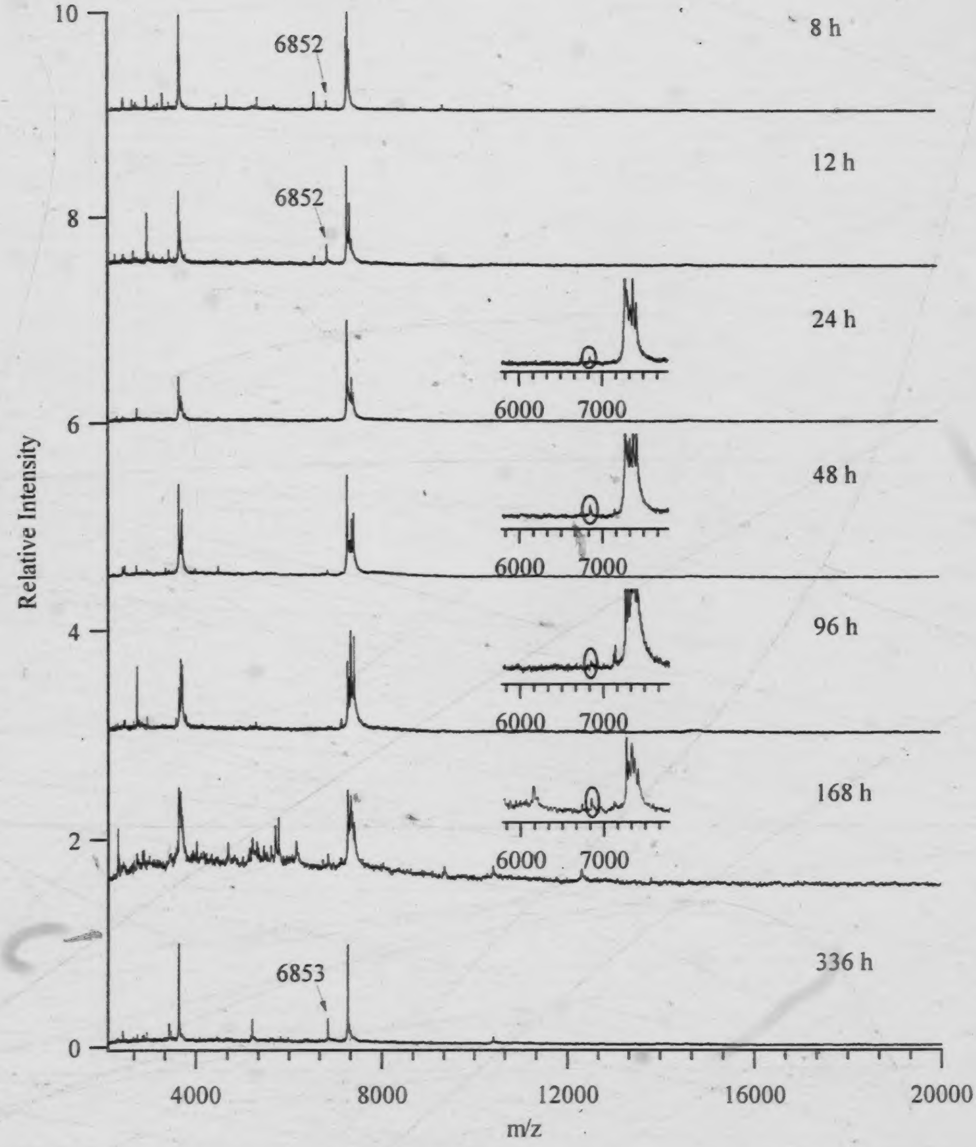

**Figure 4.5** MALDI spectra of corresponding HPLC fractions consisting of mass 6852 Da of *Bacillus megaterium* (ATCC 14581) extract.

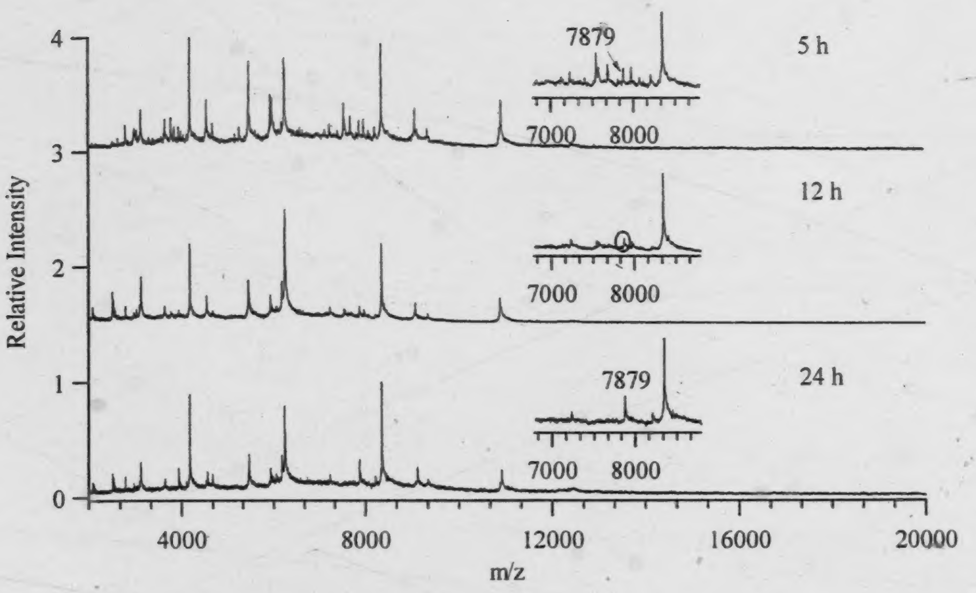

**Figure 4.6** MALDI spectra of corresponding HPLC fractions consisting of mass 7879 Da of *Streptococcus bovis* (ATCC 33317) extract.

First, the amount of protein with characteristic mass 7879 Da is low. In addition, quit a few protein entries were eluted from the column in this fraction, resulting in possible suppression. The peaks at m/z 6233 and 8352 Da may suppress the ionization of protein with characteristic mass 7879 Da. This work suggests that for those proteins with low intensity in MALDI spectra, the detection of those proteins can be more readily affected by experimental factors other than operators' experience. In an ideal case, in the creation of database for bacterial identification, parallel analysis of every sample harvested at different growth time from different labs with different operators should increase the reliability of the experimentally derived protein database.

### 4.3.2 Protein Expression Changes as a Function of Cell Growth Starter

*E. coli* (ATCC 9637) has been grown in two batches. In the first batch, it was grown from a stock of lyophilized cells (ATCC 9637) purchased from Sigma. Eight samples were collected from 8 to 48 h. The 48 h lyophilized sample from the first batch was subsequently used as the starter for cell growth of the second batch, in which twelve samples were collected from 2 to 72 h. The samples from both batches were detected by LC/ESI-MS. Table A.1 and Table A.11 (see Appendix) list the mass values of the protonated protein ions from both batches. The mass accuracy is expected to be 0.02% for the deconvoluted LC/ESI-MS data. In Table A.11, the bolded mass values correspond to those observed in both batches. Totally, there are 215 and 520 individual masses observed over the entire incubation period in both batches. Among them, 53 masses observed in the first batch are absent in the second batch, most of which were only detected in one of the earlier growth times (before 24 h) sample (bolded mass in the Table A.1). In addition, there are *ca*. 350 new masses detected in the second batch. 77 per cent of the number of new mass entries is only observed in one growth time sample; 14 per cent of it is observed in two growth period samples; 9 per cent of it can be observed in several (3-9) growth time samples. These results suggest that protein expression is also sensitive to growth starter. The starter from different generation can give rise to different protein profile. However, in bacterial identification, only those mass entries observed in the several growth time samples actually affect the identification result. From this work, it can be concluded that the starter used for creating experimentally derived database will not have much influence on the identification result, since the number of new mass entries detected in the several growth time samples from the second batch is not large enough.

### 4.3.3 Protein Expression Pattern

Figure 4.7 shows the total ion chromatograms of *E.coli* (ATCC 9637) cell extracts from the first batch. The shapes of the TICs for the samples harvested at 36 and 48 h resemble that of the sample collected at 8 h. More specifically, the 8, 36, and 48 h samples show a prominent peak at 10 min. This peak contains a very strong set of m/z peak of 2430 Da. In addition, the peak at 36 min is quite intense in the 8, 36 and 48 h samples. But, it is absent in the samples harvested in other growth times. The main component of this peak possesses a molecular weight of 9739 Da. The distribution of mass entries observed in the samples collected at all incubation times was shown in Figure 2.1. From Figures 2.1 and 4.7, it can be clearly seen that there is an interesting pattern that the proteins detected from the samples harvested at later growth period are similar to those from the samples collected at earlier growth period. This phenomenon is also observed in the analysis of *Staphylococcus kloosii* (ATCC 43959) cell growth (see Appendix, Table A.8). However, whether this is a reproducible phenomenon or experimental artifact remains to be seen. In order to shed some light on this interesting protein expression feature as well as to test its reproducibility, we thus carried out an experiment in which the lyophilized *E.coli* (ATCC 9637) cells taken at 48 h from the first batch was used as starter and incubated for 72 h under same condition as employed in the first batch.

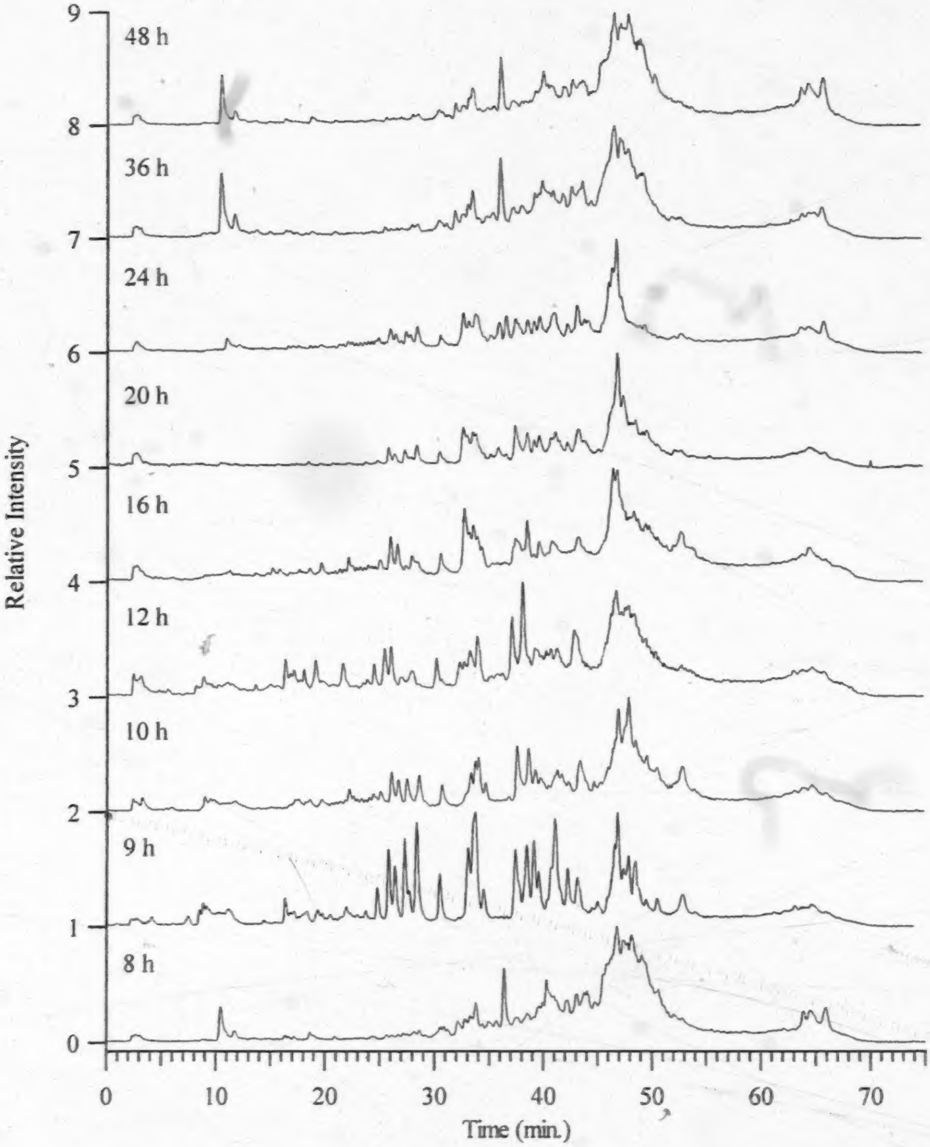

**Figure 4.7** The total ion chromatograms of *E.coli* (ATCC 9637) cell extracts from the first batch.

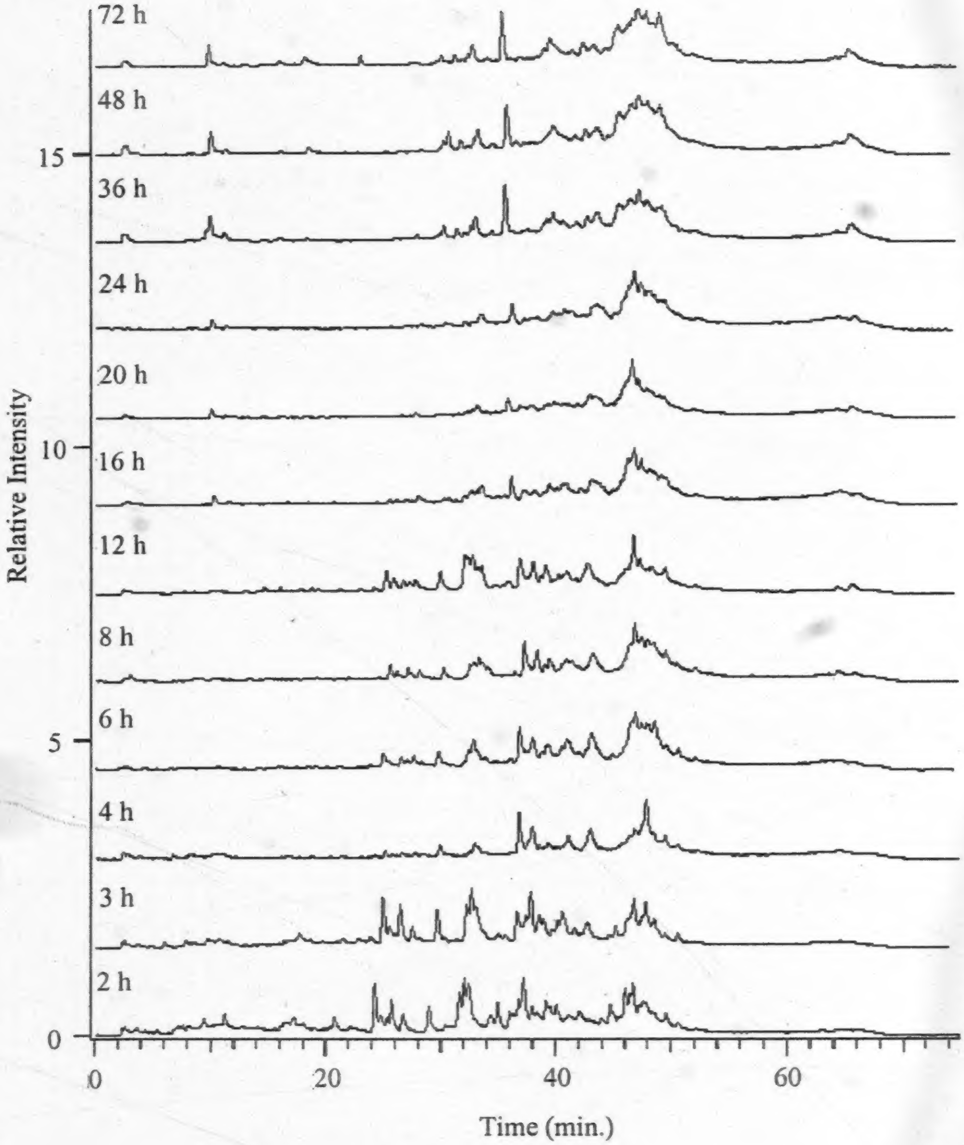

**Figure 4.8** The total ion chromatograms of *E.coli* (ATCC 9637) cell extracts from the second batch.

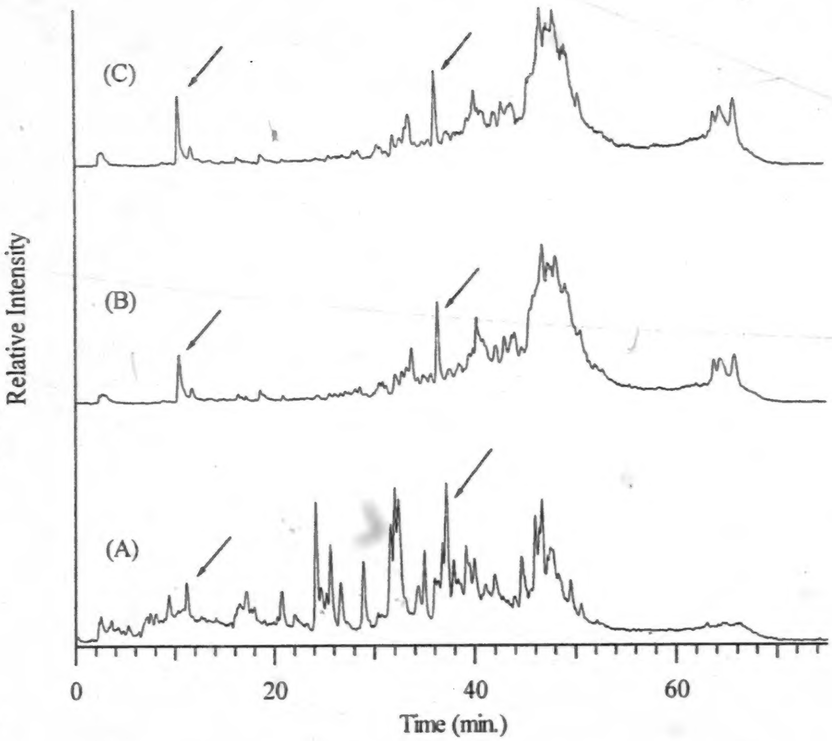

**Figure 4.9** Comparison of total ion chromatograms: (A) 2 h incubation sample of the second batch. (B) 8 h incubation sample of the first batch. (C) 48 h incubation sample of the first batch.

The cell extracts from different incubation time were subjected to LC/ESI-MS analysis, and their chromatograms are shown in Figure 4.8. Under the same analytical condition as before, however, the pattern observed in the first batch was not reproduced. From Figure 4.8 it can be seen that the shapes of TICs for the samples at 36, 48, and 72 h are quite different from that at 2 h. Table A.11 indicates in detail that the masses observed in the samples harvested in earlier growth period are not similar to those from the samples collected in later growth period.

For the first batch, the TICs of the samples from 8, 36, and 48 h showed two characteristic peaks at 10 and 36 min, corresponding to the molecules of 2,430 and 9,739

Da respectively (as shown in Figure 4.9). Even though the pattern observed in the first batch was not reproduced in the second batch, we were still able to observe these two peaks in the TIC of the 2 h sample in the second batch (see Figure 4.9). Moreover, the shapes of the TICs of the samples harvested after logarithmic growth phase become similar to those of the first batch; in particular, until 48 h both TIC results from two batches are exactly identical (see Figure 4.10 to 4.13).

## 4.4 Conclusions

In this chapter, we have demonstrated that protein expression of bacterial cells is significantly dependent on growth time and other conditions. Protein profiles observed from samples harvested at different growth phase in the cell growth curve are quite different. Also, the type of growth starter can affect the protein profile in a sensitive manner. A pattern that the proteins detected from the samples harvested in later growth period are similar to those from the samples collected in earlier growth period can be observed in the growth experiments of some bacteria, such as *E.coli* (ATCC 9637) and *Staphylococcus kloosii* (ATCC 43959). This pattern is irreproducible in the further cell growth where the sample harvested in the first batch is used as the starter of the second batch. Even though the starters in both batches are different, the shapes of the TICs after 20 h incubation are still similar.

This study suggests that the factors of various growth conditions shall be taken account in bacterial identification. This is critical for the successful identification of unknown bacteria that are produced under different growth environments. In the process of creating a statistical algorithm for bacterial identification, the factor of protein expression should be considered. The mass detected over the entire growth period

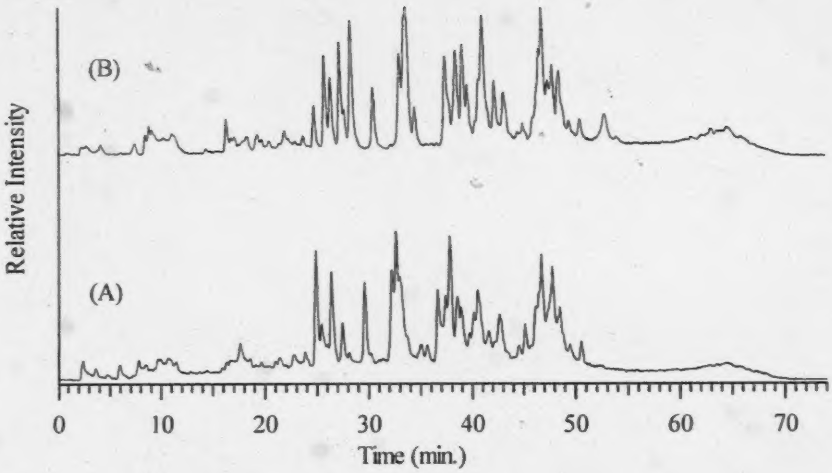

**Figure 4.10** Comparison of total ion chromatograms: (A) 3 h incubation sample of the second batch. (B) 9 h incubation sample of the first batch.

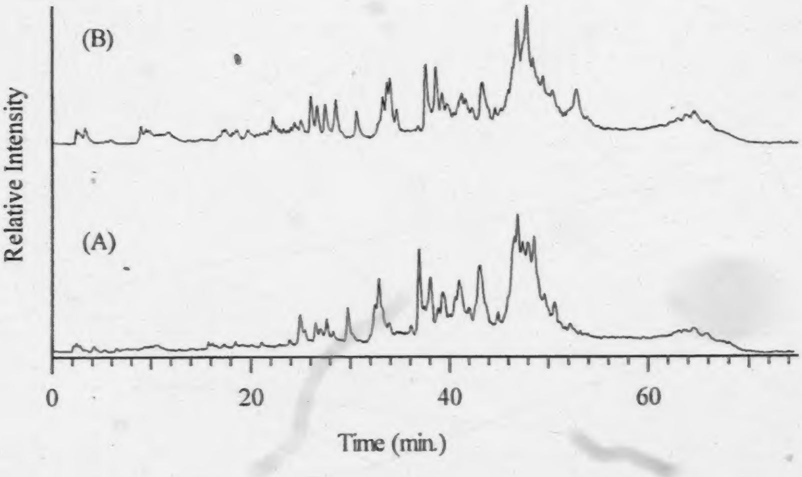

**Figure 4.11** Comparison of total ion chromatograms: (A) 6 h incubation sample of the second batch. (B) 10 h incubation sample of the first batch.

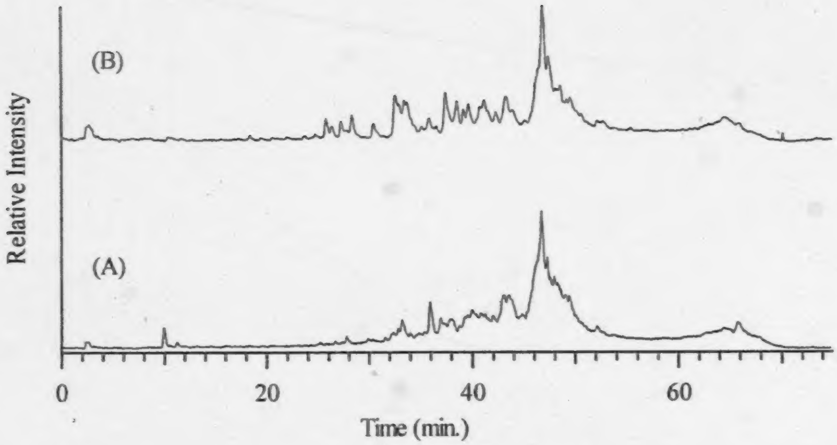

**Figure 4.12** Comparison of total ion chromatograms: (A) 20 h incubation sample of the second batch. (B) 20 h incubation sample of the first batch.

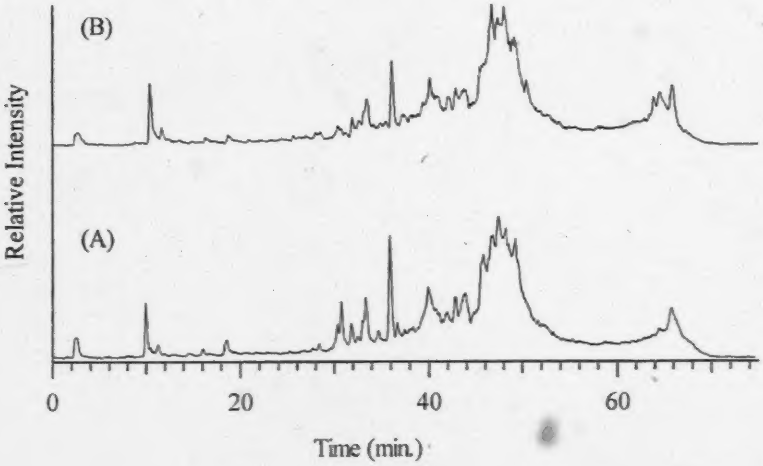

**Figure 4.13** Comparison of total ion chromatograms: (A) 48 h incubation sample of the second batch. (B) 48 h incubation sample of the first batch.

always with high intensity is a characteristic mass for respective bacterium, and should be given a heavy factor in the identification. In addition, a better statistical algorithm should reflect major changes of protein expression associated with many microbiological variations.

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# Chapter 5

# Bacterial Identification by Protein Mass Mapping Combined with an Experimentally Derived Protein Mass Database[a]

## 5.1 Introduction

As mentioned in previous chapters, the protein mass mapping approach for bacterial identification is a very promising method due to many advantages such as rapidity and versatility [1]. The general idea of the identification process can be summarized as: a set of protein masses in a mass spectrum from an unknown species is compared to an experimentally derived protein mass database. Based on the work reported in Chapter 3, a prototype of experimentally derived protein database has been created including nine bacteria species, namely, *E. coli*, *Bacillus globigii*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus arlettae*, *Staphylococcus kloosii*, *Streptococcus bovis*, *Microbacterium saperdae*, and *Pseudomonas fluorescens*. Also, we were expecting that bacterial identification using such a database would be reliable and positive.

In order to validate this protein mass mapping approach, it is imperative to perform a "blind test" using several unknown samples without knowing any identity information to operators. In this work, a numerical method using a statistical weight factor algorithm for bacterial matching and identification is introduced and applied.

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[a] Ms. Xinlei Yu and the author contributed equally to this work because of the bulk results of the data collected.

Detailed investigations with regard to the applicability, reliability and sensitivity of the protein mass mapping approach are also discussed.

## 5.2 Experimental

### 5.2.1 Chemicals and Materials

Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, MD, provided all seven unknown samples A-G for a single blind study.

In the sensitivity study, *E. coli* (ATCC 47076) was incubated in Nutrient broth at 37 °C with constant shaking. The cells were harvested at 24 h and were subsequently washed with MQ water once, lyophilized and stored at –25 °C until analysis. *Bacillus subtilis* (ATCC 23857) was grown in Brain Heart Infusion broth at 26 °C with constant shaking. The cells were harvested at 15 h and prepared as described above.

Nutrient broth and Brain heart infusion broth were from Becton Dickinson (Becton Dickinson and Company, Sparks, Maryland, USA). Spectrophotometric grade trifluoroacetic acid (TFA), $\alpha$-cyano-4-hydroxycinamic acid (HCCA), horse cytochrome *c* and Tris were purchased from Sigma-Aldrich-Fluka (Oakville, Ontario, Canada).

### 5.2.2 Sample Preparation

For each unknown sample, proteins were extracted with vortex in 50 mM Tris-HCl buffer for 2 min. The suspension was then centrifuged. The supernatant solution was used for MALDI-MS analysis. For HPLC ESI-MS analysis, proteins were extracted from unknown sample **A-F** as described in Section 3.5.2.2 (see Chapter 3). For unknown sample **G**, protein was extracted by the same method as described in Section 3.2.2.2 (see Chapter 3).

In the sensitivity study, Protein extracts from *E. coli* (ATCC 47076) and *Bacillus subtilis* (ATCC 23857) lyophilized cells were prepared by the method as described in the Section 3.1.2.2 and 3.4.2.2 respectively.

### 5.2.3 Mass Spectrometry

**MALDI.** MALDI experiments were performed using a Bruker REFLEX III matrix-assisted laser desorption/ionization time-of-flight mass (MALDI/TOF) spectrometer (Framingham, MA) on each individual fraction. Desorbed protein ions were produced with a 337 nm pulsed nitrogen laser and detected in the linear mode of operation in TOF. A two-layer sample preparation method with HCCA as the matrix was used for MALDI analysis. In the two-layer method, the first layer was formed by applying 0.8 μL of 0.1 M HCCA in methanol/acetone (3/7, v/v) to the MALDI plate. For the second layer, the sample solution was mixed in 1:8 and 1:15 ratios with saturated HCCA in formic acid/acetonitrile/water in a 1: 2: 3 by volume; 0.5 μL of the second layer solution was applied onto the first layer and allowed to dry. On-plate washing of the MALDI sample with 1 μL of distilled water was performed twice to remove the salts.

In the sensitivity study, MALDI-MS experiments were performed as described above. The two-layer deposition method combined with the micro-spot technique was employed with HCCA as the matrix in the matrix/sample preparation.

**HPLC/ESI-MS.** Before injection onto the column, a Zip Tip C4 desalted each unknown bacterial protein extract. LC was conducted on a ThermoFinnigan LCQ Deca Surveyor LC-MS system. Chromatographic separation was performed on a C8 Vydac reversed-phase capillary column (300 A, 5 μm, 150 μm i.d. × 150 mm). The mobile phase consisted of water and acetonitrile and both contained 0.5% (v/v) acetic acid. The effluent from the HPLC column was introduced to a ThermoFinnigan quadrupole ion trap MS through an ESI interface in the positive ion mode. The molecular masses of the separated proteins were determined by deconvolution of the ESI mass spectra using the Finnigan BIOMASS software.

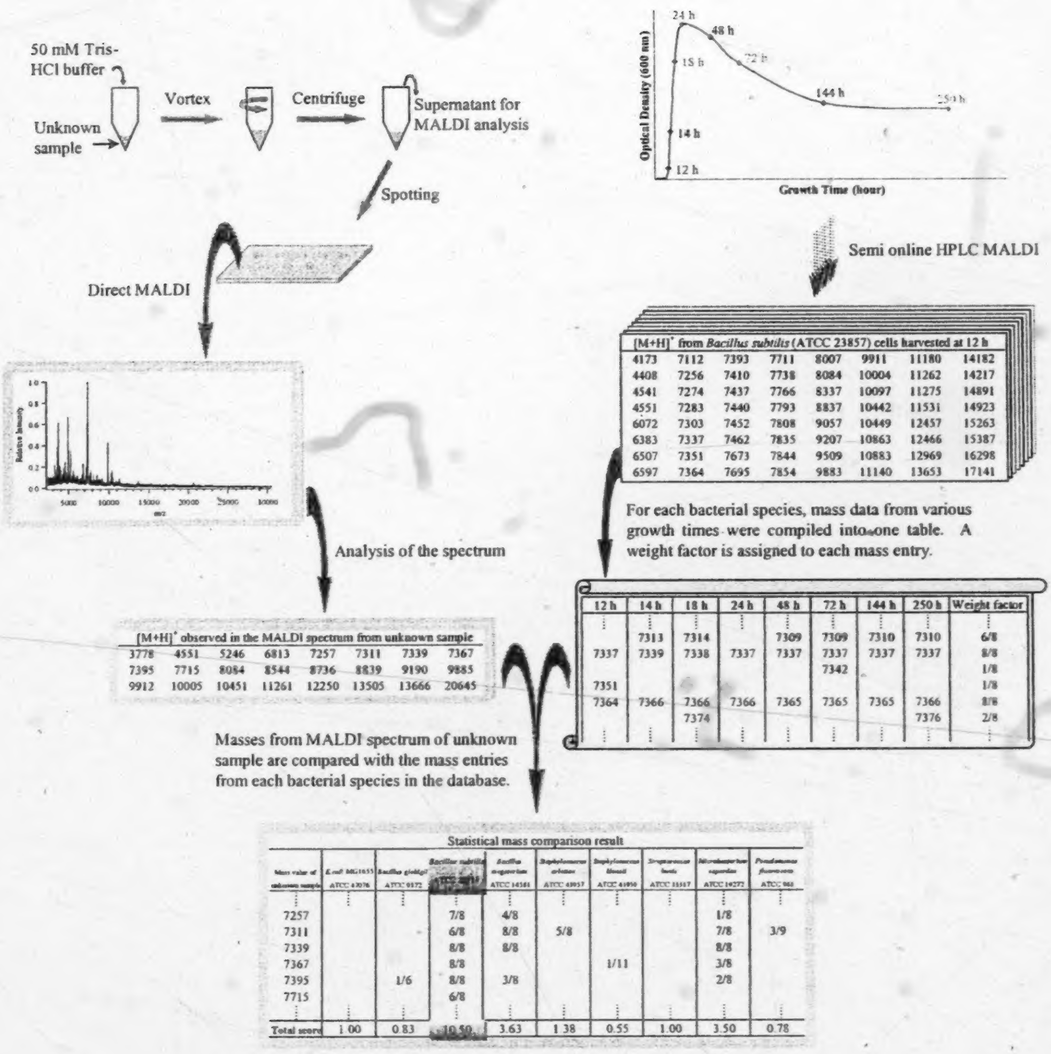
**MALDI micro-spot technique.** One centimeter of silica capillary (i.d. 50 μm) coated with bovine serum albumin was connected to the entrance of a cut-off pipette tip with a Teflon tube and used as a micro-spot pipette. The sample deposition volume was about 20 nL.

**Cell counting and calculation.** Cell counting was performed with a hemacytometer. Dry cells were diluted with MQ water and stained with 0.14% methylene blue. The cell concentration was calculated as follows: cell concentration per milliliter = total cell count in outer square × 2500 × dilution factor.

## 5.3 Results and Discussion

### 5.3.1 Statistical Weight Factor Algorithm

In bacterial identification using protein mass mapping approach, a numerical method using statistical weight factor algorithm was employed for matching unknown bacterial species against the experimentally derived protein mass database. As Figure 5.1 illustrates, after the protein mass data were generated from bacterial cells grown to varying times, these data were compiled into one table. A weight factor was assigned to an individual mass entry according to the frequency of this mass appearing at different growth times of the same bacterium. As an example, in the mass table shown in Figure


50 mM Tris-HCl buffer
Unknown sample
Vortex
Centrifuge
Supernatant for MALDI analysis
Spotting
Direct MALDI
Relative Intensity
5000
10000
15000
20000
25000
30000
m/z
Analysis of the spectrum
24 h
48 h
18 h
72 h
144 h
250 h
14 h
12 h
Optical Density (600 nm)
Growth Time (hour)
Semi online HPLC MALDI

| [M+H]⁺ from *Bacillus subtilis* (ATCC 23857) cells harvested at 12 h | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4173 | 7112 | 7393 | 7711 | 8007 | 9911 | 11180 | 14182 |
| 4408 | 7256 | 7410 | 7738 | 8084 | 10004 | 11262 | 14217 |
| 4541 | 7274 | 7437 | 7766 | 8337 | 10097 | 11275 | 14891 |
| 4551 | 7283 | 7440 | 7793 | 8837 | 10442 | 11531 | 14923 |
| 6072 | 7303 | 7452 | 7808 | 9057 | 10449 | 12457 | 15263 |
| 6383 | 7337 | 7462 | 7835 | 9207 | 10863 | 12466 | 15387 |
| 6507 | 7351 | 7673 | 7844 | 9509 | 10883 | 12969 | 16298 |
| 6597 | 7364 | 7695 | 7854 | 9883 | 11140 | 13653 | 17141 |

For each bacterial species, mass data from various growth times were compiled into one table. A weight factor is assigned to each mass entry.

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h | Weight factor |
|---|---|---|---|---|---|---|---|---|
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |
| | 7313 | 7314 | | 7309 | 7309 | 7310 | 7310 | 6/8 |
| 7337 | 7339 | 7338 | 7337 | 7337 | 7337 | 7337 | 7337 | 8/8 |
| | | | | | 7342 | | | 1/8 |
| 7351 | | | | | | | | 1/8 |
| 7364 | 7366 | 7366 | 7366 | 7365 | 7365 | 7365 | 7366 | 8/8 |
| | | 7374 | | | | | 7376 | 2/8 |
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |

| [M+H]⁺ observed in the MALDI spectrum from unknown sample | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3778 | 4551 | 5246 | 6813 | 7257 | 7311 | 7339 | 7367 |
| 7395 | 7715 | 8084 | 8544 | 8736 | 8839 | 9190 | 9885 |
| 9912 | 10005 | 10451 | 11261 | 12250 | 13505 | 13666 | 20645 |

Masses from MALDI spectrum of unknown sample are compared with the mass entries from each bacterial species in the database.

Statistical mass comparison result

| Mass value of unknown sample | *E. coli* MG1655<br>ATCC 47076 | *Bacillus globigii*<br>ATCC 9372 | *Bacillus subtilis*<br>[illegible] | *Bacillus megaterium*<br>ATCC 14581 | *Staphylococcus* [illegible]<br>ATCC [illegible] | *Staphylococcus* [illegible]<br>ATCC [illegible] | *Streptococcus* [illegible]<br>ATCC [illegible] | [illegible]<br>ATCC [illegible] | *Pseudomonas* [illegible]<br>ATCC [illegible] |
|---|---|---|---|---|---|---|---|---|---|
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |
| 7257 | | | 7/8 | 4/8 | | | | 1/8 | |
| 7311 | | | 6/8 | 8/8 | 5/8 | | | 7/8 | 3/9 |
| 7339 | | | 8/8 | 8/8 | | | | 8/8 | |
| 7367 | | | 8/8 | | | 1/11 | | 3/8 | |
| 7395 | | 1/6 | 8/8 | 3/8 | | | | 2/8 | |
| 7715 | | | 6/8 | | | | | | |
| ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ | ⋮ |
| Total score | 1.00 | 0.83 | 10.50 | 3.63 | 1.38 | 0.55 | 1.00 | 3.50 | 0.78 |

**Figure 5.1** Workflow of the protein mass mapping approach combined with experimentally derived protein mass database for bacterial identification.

5.1, the protein mass 7337 Da is observed 8 times out of the 8 growth times. Thus this mass is assigned a weight factor of 8/8. If this protein mass entry is also observed in the table for another bacterial species **Y** (data not shown) and detected 2 times out of the 8 growth times, a weight factor of 2/8 is given to 7337 Da for bacterial species **Y**.

In applying the experimentally derived protein mass database for identification of an unknown bacterial species (see Figure 5.1), mass spectrum from the unknown sample is first obtained and the protein masses are determined. Each protein mass observed in the experimental mass spectrum is compared with the mass entries from each known bacterium species in the database. The weight factor of each matched mass is listed in a column corresponding to a known bacterium in the database. The weight factors for all the database-matched masses are added to yield a summed weight factor score for an individual bacterium. The bacterium species with the highest summed weight factor score is thus deemed as the identity of the unknown sample under analysis. In some circumstances where an unknown species is not included in the database and/or for a mixture of different bacteria, an ambiguous result could be obtained where all of the summed weight factor scores would be of similar values (*vide infra*).

### 5.3.2 Applicability and Reliability of Protein Mass Mapping Approach

The protein mass mapping approach based on the use of the experimentally derived database and the statistical weight factor algorithm for matching scoring were first applied to identify bacteria grown under various different conditions including different media and grown times than those used for creating the database. In all cases, an "unknown" bacterium could be positively identified as long as it is listed in the database. The applicability and reliability of this method was then challenged in a single blind study where seven unknown bacterial samples coded as **A-G** were investigated. Both direct MALDI-MS and HPLC ESI-MS were used to analyze protein masses from protein extracts of the single blind bacteria. Representative results from unknown samples **G** and **E** are discussed below to highlight the salient features of this approach. The identification results of the other samples can be found elsewhere [2].

It is known that MALDI-MS can rapidly produce protein mass data with high sensitivity [3-4]. However, the type of proteins detected can be affected by several experimental parameters and microbiological factors including bacterial growth times, protein extraction solvent, protein extraction method, and sample/matrix solvent [5-8]. Since the database was created using a limited number of experimental conditions, it is important to ascertain the experimental parameter inclusiveness of this protein mass mapping method. To address this issue, we acquired a number of spectra of unknown samples under various experimental conditions. Several considerations can be noted: 1) the growth conditions used to prepare the single blind samples were independently chosen by a researcher in a microbiology lab at ECBC, 2) the vortex method was selected as a rapid protein extraction method for the unknown samples while sonication and French Press were used to obtain protein extracts in the creation of the database, and 3) the second-layer solution in MALDI sample preparation was composed of saturated alpha-cyano-4-hydroxycinnamic acid (HCCA) in 17% formic acid/33% acetonitrile/50% water (v/v) for the unknown samples which was found to be optimal for complicated protein mixture analysis, and this solution was not used in the creation of the database. In addition, mass spectral pattern of an unknown sample can vary to some extent with respect to the different protein concentrations used and the different MALDI sample spots interrogated. In our investigation of the effects of experimental parameters on the applicability of the mass mapping method, we took two aliquots from a bacterial extract and mixed them with the second-layer solution in 1:8 and 1:15 (v/v) ratios, respectively. Each aliquot/matrix mixture was deposited as two spots on the MALDI target. Thus a total of four mass spectra for each unknown sample were obtained.

Table 5.1 lists the protein mass matching results from one of the four MALDI mass spectra of unknown sample G obtained by using the sample/matrix ratio of 1:8 and deposition spot 1. Sample **G** produces the highest matching score of 10.50 with *Bacillus subtilis*. A summary of the matching scores for sample **G** from four different MALDI conditions is shown in Figure 5.2(A). Among the four mass spectra obtained, there are some small variations in the number of peaks observed. However, these minor variations do not affect the identification result, as shown Figure 5.2 where the highest scores in all cases are for *Bacillus subtilis*. After the result submission, it was revealed that this sample was indeed from *Bacillus subtilis*.

Figure 5.2(B) illustrates the protein mass mapping results of unknown sample **E**. An unambiguous match is not observed, because the scores for every bacterial species are very similar and low. This result suggests that unknown sample **E** could be a bacterial species not included in our experimentally derived mass database. Alternatively, the sample **E** extract might contain proteins from more than one bacterial species, although this was unlikely because of the similarly low scores found across the entire bacterial spectrum. Since the blind test was designed for identifying single bacterial species, we concluded that sample **E** was a bacterial species not listed in the database. Sample E was

**Table 5.1** Protein mass mapping results from comparing the protein masses obtained from the unknown sample G to those in the experimentally derived protein mass database.

| Mass value of unknown sample | *E.coli* ATCC 47076 | *Bacillus globigii* ATCC 9372 | *Bacillus subtilis* ATCC 23857 | *Bacillus megaterium* ATCC 14581 | *Staphylococcus arlettae* ATCC 43957 | *Staphylococcus kloosii* ATCC 43959 | *Streptococcus bovis* ATCC 33317 | *Microbacterium saperdae* ATCC 19272 | *Pseudomonas fluorescens* ATCC 948 |
|---|---|---|---|---|---|---|---|---|---|
| 3778 | | | | 2/8 | | | 2/8 | | |
| 4551 | | | 4/8 | | 2/8 | | | | |
| 5246 | | | | | 1/8 | | | | |
| 6813 | | | | | | | 2/8 | | |
| 7257 | | | 7/8 | 4/8 | | | | 1/8 | |
| 7311 | | | 6/8 | 8/8 | 5/8 | | | 7/8 | 3/9 |
| 7339 | | | 8/8 | 8/8 | | | | 8/8 | |
| 7367 | | | 8/8 | | | 1/11 | | 3/8 | |
| 7395 | | 1/6 | 8/8 | 3/8 | | | | 2/8 | |
| 7715 | | | 6/8 | | | | | | |
| 8084 | | | 1/8 | | | 1/11 | | | |
| 8544 | | | 2/8 | | | | | | |
| 8736 | | | 1/8 | | | | | | |
| 8839 | | | 3/8 | | | | | | 4/9 |
| 9190 | 6/6 | 1/6 | 3/8 | | | | | | |
| 9885 | | 1/6 | 8/8 | 2/8 | | 4/11 | | | |
| 9912 | | | 7/8 | | | | | | |
| 10005 | | 1/6 | 4/8 | | 2/8 | | 1/8 | 7/8 | |
| 10451 | | | 3/8 | 2/8 | 1/8 | | 2/8 | | |
| 11261 | | | 5/8 | | | | 1/8 | | |
| 12250 | | | | | | | | | |
| 13505 | | | | | | | | | |
| 13666 | | | 1/8 | | | | | | |
| 20645 | | 1/6 | 2/8 | | | | | | |
| **Total score** | 1.00 | 0.83 | 10.50 | 3.63 | 1.38 | 0.55 | 1.00 | 3.50 | 0.78 |

actually from *Edwardsiella tarda Ewing and Mcwhorter* (ATCC 15947) which was indeed not listed in the database.

We found that neither the protein extraction process nor the mode of detection (i.e., ESI-MS vs. MALDI) has a significant effect on the applicability of the protein mass mapping method. As an example, ESI mass spectra of unknown sample **G** were acquired in a completely different fashion (see Experimental section) from that used in the creation of the protein mass database. In this case, instead of using the French Press method for

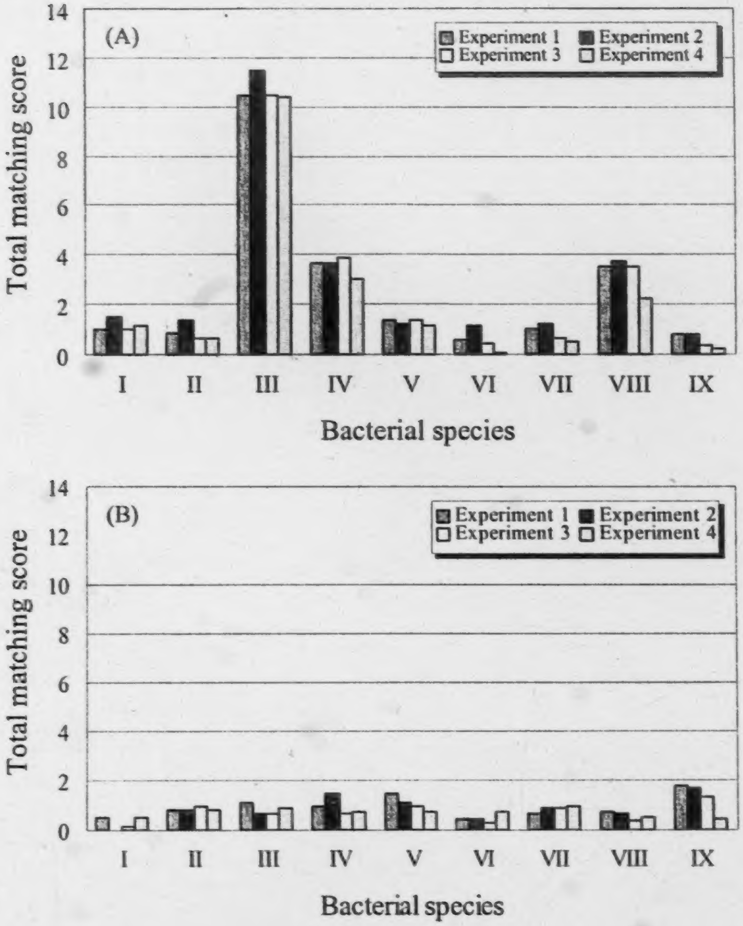


**Figure 5.2** Protein mass mapping results from (A) unknown sample G and (B) unknown sample E obtained under different experimental conditions. Samples were detected by off-line HPLC MALDI-MS from two spots where protein extract HPLC eluant aliquots were mixed with the second layer in a 1:8 ratio for experiments 1 and 2 and 1:15 for experiments 3 and 4. The names of bacterial species from I-IX on the *x*-axis are: *E. coli* (ATCC 47076), *Bacillus globigii* (ATCC 9372), *Bacillus subtilis* (ATCC 23857), *Bacillus magaterium* (ATCC14581), *Staphylococcus arlettae* (ATCC 43957), *Staphylococcus kloosii* (ATCC 43959), *Streptococcus bovis* (ATCC 33317), *Microbacterium saperdae* (ATCC 19272), and *Pseudomonas fluorescens* (ATCC 948), respectively.

**Table 5.2** Statistical mass comparison of $[M+H]^+$ (Da) from unknown sample **G** extract by LC/ESI-MS to experimentally derived protein mass database

| Mass value of unknown sample | *E. coli* ATCC 47076 | *Bacillus globigii* ATCC 9372 | *Bacillus subtilis* ATCC 23857 | *Bacillus megaterium* ATCC 14581 | *Staphylococcus arlettae* ATCC 43957 | *Staphylococcus kloosii* ATCC 43959 | *Streptococcus bovis* ATCC 33317 | *Microbacterium saperdae* ATCC 19272 | *Pseudomonas fluorescens* ATCC 948 |
|---|---|---|---|---|---|---|---|---|---|
| 3888 | | | | | | | | | |
| 5156 | | | | | | 1/11 | 1/8 | | |
| 7115 | 1/6 | | 2/8 | | | | | | |
| 7613 | | | | | | | | | 4/9 |
| 7639 | | | | | 1/8 | | 1/8 | 1/8 | 9/9 |
| 10868 | 1/6 | | 6/8 | | | 1/11 | 2/8 | | |
| 11267 | | | 5/8 | | | | 4/8 | | |
| 12973 | | | 3/8 | | | | | | |
| 15292 | | | | | | | | | |
| 16953 | | | 5/8 | | | | | | |
| 17075 | | | | | | | | | |
| 17148 | | | 1/8 | 2/8 | | | | | |
| 17337 | | | 3/8 | | | | | | |
| 20781 | | | | 1/8 | | | | | 1/9 |
| **Total score** | 0.33 | 0.00 | 3.13 | 0.38 | 0.13 | 0.18 | 1.00 | 0.13 | 1.56 |

cell lysis and extraction, we extracted the protein by sonication in 50 mM Tris buffer for 10 minutes. In spite of this variation and the use of different mode of mass detection, we still positively identified unknown sample **G** as *Bacillus subtilis*. Table 5.2 lists the matching scores from the protein mass data obtained by HPLC ESI-MS analysis of sample **G**. Although the masses detected by the MALDI and ESI-MS procedures are different (see Tables 5.1 and 5.2), the identification results of these two methods are the same. It appears that the HPLC MALDI-MS generated database is versatile in accommodating datasets from different MS techniques for bacterial identification.

In the single blind test, the identification results of all seven unknown samples were found to be *correct*. This result of 100% accuracy substantiates that the protein mass mapping method described in this work has significant potential for rapid and effective bacterial identification.

### 5.3.3 Sensitivity Test

Many studies have shown that MS techniques such as direct MALDI analysis of cell extracts [1, 9-17] or whole cell lysates [6-8, 18-28] can be relatively rapid and respond to very low amounts of sample [29-31]. Nevertheless, the detection limit for unambiguous bacterial identification based on protein mass mapping is a parameter that still needs investigation. In the determination of the detection sensitivity of the bacterial identification process, cell extracts of *E. coli* and *Bacillus subtilis*, representing Gram-negative and Gram-positive cells, respectively, were used as model systems. Each bacterial sample was grown in a different broth from those used for bacterial growth in the creation of the database.

Protein extracts from bacterial cells can be directly subjected to MALDI-MS analysis without further sample preparation processes, and this reduces the possibility of protein loss. Our group has developed an efficient micro-spotting sample deposition method for analyzing trace amounts of protein samples including the analysis of proteins from a single cell [32]. By using this method, the sample/matrix (1:1 by volume) deposition volume is reduced from 0.5 μL to 20 nL, and the sample spot is reduced from 2 mm to less than 0.5 mm in diameter. When a laser beam diameter of less than 150 microns is used to desorb and ionize the protein sample in a micro-spot, the majority of the proteins can be consumed. Sample utilization efficiency is thus increased, providing an increase in overall detection sensitivity, which can translate into an improvement in the detection limit for bacterial identification.

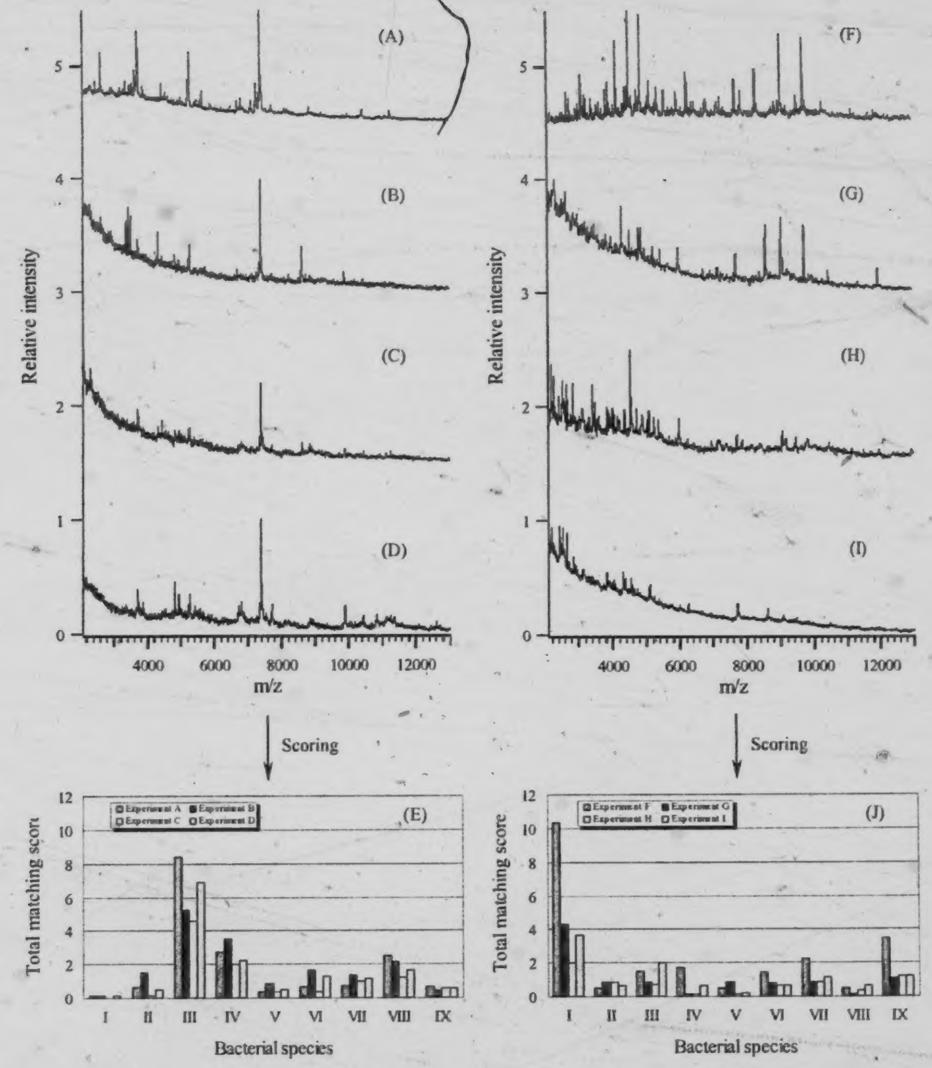


**Figure 5.3** MALDI mass spectra of *Bacillus subtilis* (ATCC 23857) obtained by using conventional sample deposition of a protein extract at a concentration of (A) 200 ng/µl, (B) 55 ng/µl, (C) 24 ng/µl. (D) mass spectrum obtained by using microspot sample deposition of a protein extract at a concentration of 24 ng/µl and (E) its corresponding protein mass mapping results. MALDI mass spectra of *E. coli* (ATCC 47076) obtained by using conventional sample deposition of a protein extract at a concentration (F) 500 ng/µl, (G) 25 ng/µl, (H) 13 ng/µl. (I) mass spectrum obtained by using microspot sample deposition of a protein extract at a concentration of 13 ng/µl and (J) its corresponding protein mass mapping results.

Figure 5.3 shows the results of the sensitivity study and the comparison of conventional sample loading vs. microspot loading. It is clear that the lower the protein extract concentration, the fewer the number of protein peaks observed. There is a threshold concentration under which a sufficient number of proteins are no longer detected for reliable protein mass mapping. Using the microspot sample deposition method, the protein concentration can be kept high, yet it requires less amount of sample due to the use of nanoliter for sample deposition vs. microliter in conventional sample preparation method. The protein masses detected from the MALDI spectra shown in Figure 5.3(D) and Figure 5.3(H) are sufficient for identification of *Bacillus subtilis* and *E. coli*, respectively. These experimental results show that the detection limit for the identification of *Bacillus subtilis* by MALDI-MS combined with the micro-spot technique is *ca*. 480 pg of proteins, and this corresponds the amount of proteins that can be extracted from *ca*. 410 ± 80 dry cells (the error comes from replicate experiments involved in studying the relation of protein amounts of cell extracts and cell counts). For *E. coli*, this detection limit is *ca*. 260 pg of proteins and corresponds to *ca*. 650 ± 40 dry cells. This work demonstrates that the protein mass mapping method with microspot MALDI detection has the sensitivity for identifying bacterial samples with less than 1000 cells. Future work will focus on the development of an on-line cell concentration, cell lysis, protein extraction, and microspot sample preparation technique tailored to bacterial identification.

## 5.4 Conclusions

We have demonstrated that the protein mass mapping approach combined with the experimentally derived protein mass database is widely applicable and reliable for rapid and positive identification of bacterial samples. Bacterial samples can be identified at the species level with satisfactory accuracy and efficiency. The major advantages of this protein mass mapping approach, compared to other methods such as peptide fragment ion spectral searching against a proteome sequence database, include its relative speed with which to analyze data, apparent simplicity of the experiments involved, not dependence on the availability of genome or proteome sequence information, and remarkable sensitivity. Although our experimentally derived protein mass database tables were generated under a relatively limited set of microbial and analytical experimental parameters, for the identification of unknown samples, there was no particular restriction on experimental factors such as bacterial growth times and cultures, protein extraction solvents, protein extraction methods, and sample/matrix solvents. The protein mass database created by using off-line HPLC MALDI-MS is somewhat independent of analytical methods, because it was proven amenable to HPLC ESI mass spectra. To date, the database only contains a small number of bacteria; but it can be readily expanded to include any bacteria of interest and tailored to a specific application. We envision that many procedures involved in database creation and unknown sample analysis can be automated. A fully automated system based on protein mass mapping for bacterial identification appears to have the potential to become a powerful tool in many real world applications such as in homeland security and in biological defense where rapid monitoring of a spectrum of bacterial agents is required.

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# Chapter 6

## Conclusions and Prospects

The ultimate goal of the research as described in this thesis is targeted at the implementation of an experimentally derived protein mass database into a practical and applicable methodology for rapid and accurate bacterial identification. In this work, we have described the feasibility, applicability, and advantages of this newly emerged MS-based bacterial identification approach through a series of extensive and in-depth studies. Our current results indicate that this protein mass mapping method, with a great possibility, will be developed into a comprehensive and reliable approach alternative to many currently used bacterial identification techniques.

The work in Chapter 2 has demonstrated and verified the strategy and protocol for the creation of this experimentally derived mass spectral database. In theory, the method used to generate the database is able to include detail microbiological parameters and post-translation information; as a result, providing better accuracy and reliability in bacterial identification.

In Chapter 3, we have constructed a prototype experimentally derived mass database by applying the protocol established in Chapter 2. To date, nine bacterial species, namely, *E. coli*, *Bacillus globigii*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus arlettae*, *Staphylococcus kloosii*, *Streptococcus bovis*, *Microbacterium saperdae*, and *Pseudomonas fluorescens* have been successfully included in the database. At current stage, microbiological factor of varied growth time is coherently introduced by compiling a set of protein mass tables for each bacterial species determined at different growth time.

The pattern of low mass protein entries in the mass spectra proves to be an effective measure of the variation of protein expression. Our study described in Chapter 4 shows that protein expression in bacterial cells is dependent upon the growth time in a very sensitive manner. Also, changes of other experimental conditions, *e.g.*, type of starter, can also influence the protein expression. This observation, accordingly, suggests that such factors as varied experimental conditions need to be considered and evaluated with caution in further database completion work. This also presents a challenge in our future works, that is, how to apply a reasonable and logical statistical algorithm to accurately identify bacterial species with all these experimental parameters under appropriate consideration. Basically, the algorithm should reflect the major changes of protein expression associated with microbiological variation.

In Chapter 5, we have demonstrated that the protein mass mapping approach combined with the experimentally derived protein mass database is widely applicable and reliable for the rapid and positive bacterial identification. The major advantages of the method we reported include its relatively fast data acquiring and analyzing speed, apparent simplicity of the experiments involved, not dependence on the availability of genome or proteome sequence information, and remarkable sensitivity. In the identification of unknown samples, there was no particular restriction on experimental factors and analytical methods.

Obviously, the database we created so far is still in a very limited scope and need to be expanded for practical applications. As envisioned, a real overall database must take not only the growth time but also other factors into account, such as ecological environment, detection MS techniques, parallel analysis of each sample harvested at different period, interlaboratory comparison, and so on. Upon this consideration, the more comprehensive the database we create, the more accurate and reliable identification result it will give. The current statistical algorithm used for bacterial identification is just a prototype model based on a database with limited bacteria species. To date, our goal is to test the reliability and applicability of the protein mass mapping approach and positive result has been obtained. With the expansion of experimentally derived protein mass database, the future work will also target on building up improved statistical algorithm model suitable for more comprehensive database and for computerized automation of data processing and identification. Eventually, a fully established bacterial protein MS database in conjunction with an automated data processing and analyzing system will become a powerful tool in many future microbiology related applications.

# Appendix

**Table A.1** $[M+H]^+$ (Da) from *E. coli* (ATCC 9637) extracts by LC/ESI-MS*.

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| 2388.9 | | | | | | 2388.8 | 2388.5 | 2388.6 |
| 2430.8 | | | | | 2430.5 | 2430.8 | 2430.8 | 2430.8 |
| 2444.9 | | | | | | | 2445.0 | 2445.0 |
| 2453.1 | | | | | | | 2453.1 | 2452.6 |
| **2468.5** | | | | | | | | |
| 2472.9 | | | | | 2472.7 | 2472.9 | 2472.9 | 2472.8 |
| **2514.3** | | | | | | | **2514.8** | |
| **3038.2** | | | | | | | | |
| | | | | | | | | **3081.7** |
| **3122.0** | | | | | | | | |
| | 3412.4 | 3412.4 | 3412.7 | | | | | |
| | | | **3921.2** | | | | | |
| | 4332.9 | 4331.9 | 4331.7 | 4332.1 | 4330.2 | 4332.0 | | |
| | 4364.0 | 4362.4 | 4362.3 | 4360.8 | | | | |
| | | | | | **4378.0** | | | |
| | **4393.8** | **4394.7** | | | | | | |
| | 4431.1 | 4432.0 | 4430.2 | | 4431.3 | | | |
| | 4460.8 | 4460.6 | 4460.3 | | | | | |
| | | | 4647.7 | | | | | |
| 5096.4 | | | | | | 5097.1 | 5095.5 | |
| | 5170.7 | 5170.7 | 5170.6 | | | 5170.6 | | |
| | 5269.8 | | 5269.1 | | | | | |
| | **5369.8** | | **5367.7** | | | | | |
| **5380.2** | | | | | | **5381.4** | | |
| | | | | 5440.6 | 5440.4 | 5440.6 | | |
| | 5884.6 | 5883.8 | 5884.1 | 5884.2 | 5883.0 | 5884.4 | | |
| | 5915.8 | 5916.4 | 5914.6 | | | | | |
| | 5946.2 | 5946.3 | | 5947.7 | | | | |
| | | | **5983.3** | | | | | |
| | 6088.4 | | | | | | | |
| **6254.5** | | | | | **6255.0** | **6256.1** | **6254.5** | |
| | 6259.6 | 6258.8 | 6258.9 | 6258.9 | 6261.0 | | | |
| | 6268.3 | | | | | | | |

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| | | **6273.9** | **6275.1** | | | | | |
| | 6291.7 | 6292.4 | | | | | | |
| 6315.1 | | | | | | 6315.1 | 6314.5 | 6315.1 |
| | 6322.4 | 6323.4 | | | | | | |
| | 6357.4 | 6358.5 | 6357.6 | 6359.1 | 6361.9 | | | |
| | **6388.3** | | | | | | | |
| | **6390.1** | | | | | | | |
| | 6425.0 | 6425.0 | 6425.0 | 6425.0 | 6424.5 | 6425.3 | | |
| | **6487.6** | | | | | | | |
| | 6524.2 | 6524.2 | 6524.1 | 6523.7 | | | | |
| | | | | | | **6583.7** | | |
| | 6609.9 | | | 6609.7 | 6609.0 | | | |
| | | | **6640.3** | | | | | |
| | 6655.6 | | 6656.1 | | | | | |
| | 6670.4 | 6670.7 | 6670.3 | | 6670.0 | | | |
| | 6684.4 | 6683.7 | 6684.3 | | | | | |
| 6695.0 | | | | | | | 6694.9 | 6694.9 |
| | **6748.1** | | | | | | | |
| | **6762.0** | | | | | | | |
| | 6768.0 | | 6770.7 | | | | | |
| | 6782.1 | 6782.4 | 6782.0 | | | | | |
| | 6792.5 | 6792.4 | 6792.3 | 6792.4 | 6791.7 | 6792.6 | | 6794.2 |
| | **6799.1** | **6799.1** | **6799.8** | | | | | |
| | **6822.3** | **6822.8** | | | | | | |
| | 6890.9 | 6890.0 | 6890.0 | 6891.2 | 6890.3 | 6890.0 | | |
| | | | | | **6988.4** | | | |
| | | | | | | | 7081.0 | |
| | 7163.1 | 7160.2 | 7161.1 | 7159.4 | 7161.6 | 7161.3 | | |
| | 7178.0 | 7178.8 | 7178.4 | | | | | |
| 7214.6 | | | | | 7215.4 | 7214.9 | | |
| | 7239.1 | 7238.9 | | | | | | |
| | | | | | 7262.8 | | | |
| 7273.6 | | | | | | | | |
| | | | | | | | 7319.6 | |
| | | | | | | | 7331.1 | |
| | 7365.9 | 7366.5 | 7365.7 | 7365.7 | 7364.8 | 7366.1 | 7365.7 | |
| | 7394.0 | 7393.8 | | | | | | |
| | **7464.0** | | | **7464.0** | | **7465.4** | | |

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| 7706.8 | | | | | | 7706.7 | 7707.3 | 7707.1 |
| 7732.2 | | 7732.2 | 7732.5 | 7732.2 | 7731.7 | 7732.4 | 7732.7 | 7732.3 |
| | 7768.3 | 7767.6 | 7768.2 | 7769.0 | 7768.3 | | | |
| | 7796.2 | | | 7795.5 | 7794.3 | 7795.9 | | |
| | | | | **7831.3** | **7831.0** | **7832.0** | | |
| | 7867.4 | | | | | | | |
| | | | | **7894.6** | | | | |
| | | | | | 8067.6 | | | |
| | | | | | | | | 8137.2 |
| 8325.5 | | | | | | | 8325.2 | 8325.1 |
| | | | **8440.9** | | | | | |
| 8545.9 | | | | | | | | 8545.3 |
| 8576.6 | | | | | | | | 8575.3 |
| | | | | | | | | **8578.0** |
| | | **8638.6** | **8638.1** | **8637.4** | | | | |
| | 8682.6 | 8682.3 | 8682.0 | 8682.6 | 8681.4 | 8682.6 | | |
| | | | | | 8725.2 | | 8725.8 | |
| | | | | 8780.8 | 8780.6 | | | |
| | | | | | 8868.7 | | | |
| 8875.8 | | | | | | | 8875.0 | |
| 8896.6 | | | | | | | 8896.5 | |
| | **8971.9** | | | | | | | |
| 8993.1 | | | | | | | | |
| 9063.6 | | | | | | 9063.8 | 9063.5 | 9063.3 |
| | 9195.3 | | | | | | 9190.8 | |
| 9211.3 | 9211.5 | 9211.8 | 9211.5 | 9211.4 | 9211.3 | 9212.2 | 9211.8 | 9212.0 |
| 9226.2 | | | | | 9225.0 | 9226.5 | 9226.2 | 9225.8 |
| | 9272.8 | | | | | | | |
| | **9275.0** | **9275.0** | | | | | | |
| | 9309.8 | 9309.6 | 9310.1 | 9308.5 | 9308.9 | | | |
| | 9312.1 | 9311.8 | | 9310.3 | 9312.9 | 9310.1 | | |
| 9322.1 | | | | | | | | 9321.0 |
| 9341.3 | 9340.8 | 9340.7 | 9341.0 | 9340.1 | 9339.9 | 9340.8 | 9341.7 | |
| | | **9380.0** | **9383.5** | | | | | |
| | 9404.0 | | | | | | | |
| | 9439.3 | 9439.2 | 9440.1 | 9441.8 | 9437.8 | | | |
| | | | | 9524.4 | | | 9523.5 | |

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| | | | | | 9531.9 | | | |
| 9534.9 | | | | | | 9535.6 | 9534.6 | 9535.9 |
| 9554.0 | | | | | | 9552.5 | 9553.0 | |
| 9592.6 | | | | 9591.5 | 9591.7 | 9592.7 | | 9592.3 |
| 9642.5 | 9642.7 | 9642.3 | | 9642.5 | 9641.7 | 9642.8 | 9642.6 | 9641.9 |
| | | | 9669.9 | | 9669.3 | 9670.3 | | |
| | | | | | **9689.7** | | | |
| | | | | | | **9706.1** | | |
| | 9729.6 | 9729.7 | 9727.5 | | 9728.5 | | | |
| 9739.3 | | | 9739.9 | | 9740.0 | 9740.5 | 9739.0 | 9738.8 |
| 9761.4 | | | | | | | 9759.1 | |
| | | | | 9770.5 | | | | |
| **9775.0** | | | | | | | | |
| 9800.4 | | | | | | 9802.6 | | 9800.0 |
| 9838.1 | | | | | | | 9837.4 | 9838.8 |
| | | 9857.0 | 9858.0 | 9857.1 | | | | |
| | | | | | 9900.0 | 9900.4 | | |
| | | | **9958.5** | | | | | |
| 9987.5 | 9986.8 | 9986.6 | 9986.6 | 9986.4 | 9985.7 | 9986.9 | | |
| 10058 | | | | | 10057 | | | |
| | 10086 | 10086 | | 10088 | 10084 | 10086 | | |
| 10299 | | | | | | | 10298 | |
| | | | | | | | 10386 | 10384 |
| | | | | 10430 | 10428 | 10429 | | |
| | | | | | | | | 10464 |
| 10496 | 10496 | 10496 | 10496 | 10497 | 10496 | 10497 | 10496 | 10495 |
| | | | **10516** | | | | | |
| | **10558** | **10560** | | | | | | |
| | 10596 | 10595 | | 10596 | | | | |
| 10651 | | | | | | | | |
| 10693 | | | | | | | 10694 | |
| 10750 | | | | | | | | |
| | | 10934 | | 10935 | 10933 | | | |
| | | | **11109** | | | | | |
| | 11119 | 11118 | | | | | | |
| 11186 | | | | | | 11185 | 11184 | |
| 11229 | 11229 | 11229 | 11229 | 11228 | 11227 | 11228 | | 11227 |
| | 11252 | | | | 11250 | | | |

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| | 11291 | | | | | 11292 | | |
| | 11328 | 11326 | | 11330 | | 11326 | | |
| | 11345 | 11345 | | | | 11345 | | |
| 11449 | | | | | | | 11448 | 11448 |
| 11553 | 11552 | 11553 | | | 11552 | | 11553 | 11552 |
| | | | **11747** | | | | | |
| 11978 | | | | | | | | |
| 12226 | | | | | | | 12227 | |
| | **12386** | | | | | | | |
| | 12517 | 12517 | | | 12517 | | 12517 | |
| 12535 | 12536 | 12535 | 12535 | 12536 | 12534 | 12536 | 12533 | 12535 |
| | 12545 | | | | | | | |
| | 12634 | 12633 | | | | | | |
| 12771 | | | | | | | | |
| | 13106 | 13106 | | 13105 | 13105 | 13106 | | |
| 13319 | 13120 | 13121 | | | 13119 | | 13121 | |
| | | | | | 13165 | | 13167 | |
| | 13205 | 13206 | | | 13204 | | | |
| | 13319 | | | | 13319 | | 13319 | |
| | | | | 13481 | 13479 | | | |
| | 13623 | 13622 | | 13622 | 13621 | 13623 | | |
| | 13653 | 13653 | | | | 13653 | | |
| | 13722 | 13722 | | | | 13723 | | |
| | **13757** | | | | | | | |
| | 14359 | 14359 | 14359 | 14359 | 14358 | 14360 | 14360 | 14359 |
| | 14459 | 14458 | | | 14457 | 14460 | | |
| | | | | | 14750 | | 14749 | |
| | 14971 | 14970 | | | | | | |
| | **15001** | | | | | | | |
| | 15315 | 15314 | | 15314 | 15313 | 15316 | | |
| | **15344** | | | | | **15346** | | |
| | **15377** | | | | | | | |
| 15409 | 15415 | 15415 | 15415 | | 15412 | 15413 | 15407 | 15408 |
| | **15446** | | | | | | | |
| | 15492 | 15492 | | | 15491 | 15492 | | |
| | 15589 | | | | | | | |
| 15690 | | | | | | | | |
| | | | | | **15748** | **15750** | | |

| 8 hr | 9 hr | 10 hr | 12 hr | 16 hr | 20 hr | 24 hr | 36 hr | 48 hr |
|---|---|---|---|---|---|---|---|---|
| 15933 | | | | | | | 15931 | |
| | 16065 | 16065 | | | | | | |
| | | | | | **16198** | | | |
| | | | | | 16370 | | | |
| | 16428 | 16428 | 16429 | | 16426 | 16428 | 16426 | |
| | | | | | 16709 | | | |
| 16934 | | | | | | | | 16933 |
| | | | | | | 17026 | | |
| | | | | | | | 17515 | |
| 17530 | 17530 | 17530 | | | | | 17530 | |
| | 17767 | 17764 | | | 17763 | 17766 | 17768 | |
| | | | | | | **17778** | | |
| | 17905 | | | | | | | |
| 17957 | | | | | | | | |
| 18160 | | | | | | 18160 | 18161 | 18162 |
| | | | | | | | 18563 | |
| | | | | | | | 18772 | |
| 18931 | | | | | 18932 | 18932 | | |
| | | | | **18982** | | | | |
| | | | | 19093 | 19090 | 19093 | | |
| 19878 | | | | | 19875 | | 19878 | |
| | 20164 | | | | 20163 | | | |
| | 22561 | 22558 | | | | | | |
| | **22661** | | | | | | | |
| **24962** | | | | | | | | **24965** |
| | 29775 | 29776 | | | | | | |
| | 31094 | 31095 | | | | | | |
| 35171 | | | | | | | 35169 | 35172 |
| | | | | **35551** | | | | |
| | | | | | | | | 35914 |

* Bolded masses correspond to those observed in the first batch but absent in the second batch.

**Table A.2** $[M+H]^+$ (Da) from *E. coli* (ATCC 47076) extracts by LC/off-line MALDI MS*.

| **2 h** | **4 h** | **12 h** | **24 h** | **48 h** | **72 h** |
|---|---|---|---|---|---|
| **2015** | | | | | |
| **2207** | | | | | |
| | | | | | **2222** |
| | | | | | 2239 |
| | | | | | **2254** |
| **2299** | | | | | |
| | **2348** | | | | |
| | 2370 | | | | |
| **2389** | **2390** | | | | |
| | **2410** | | | | |
| **2416** | | | | | |
| | | **2454** | | | |
| | | **2467** | **2467** | | |
| | | | | | **2554** |
| 2644 | | | | | |
| | | | | | 2814 |
| **2869** | | | | | |
| | | | | **3100** | |
| | | | **4048** | **4047** | |
| | | | | **4063** | |
| 4366 | 4364 | | | | |
| | **4579** | | | | |
| **4583** | | | | | |
| | **4989** | | | | |
| | **5050** | | | | |
| | | | **5092** | | |
| | | 5098 | | | |
| | | | **5153** | | |
| | | | **5207** | | |
| | | | | 5295 | |
| | 5304 | | | | |
| | | | | **5322** | |
| | **5382** | **5384** | **5382** | | |
| | **5410** | **5410** | | | |
| | | **5754** | **5754** | | |
| | | **5832** | | | |
| 5843 | | | | | |

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| | | **5854** | | | |
| | | **5874** | | | |
| | **6045** | | | | |
| | **6065** | | | | |
| | **6160** | | | | |
| **6256** | **6256** | **6256** | **6256** | **6256** | **6256** |
| | | **6260** | | | |
| | | | **6275** | | |
| 6316 | 6315 | 6317 | 6316 | 6317 | |
| | 6387 | | | | |
| 6413 | | | | | |
| | | | | 6503 | |
| | | 6508 | | | |
| | **6829** | | | | |
| 6858 | 6857 | 6857 | 6856 | 6859 | 6856 |
| | | | | **6873** | |
| | | | 6946 | 6950 | 6948 |
| | | **7112** | | | |
| | | | **7148** | | |
| | | 7151 | 7151 | | |
| 7274 | 7272 | 7272 | 7272 | 7274 | 7273 |
| | | | | | 7297 |
| 7334 | 7333 | 7333 | 7332 | 7334 | 7333 |
| 7405 | | | | | |
| | **7430** | | | | |
| 7466 | | | | | |
| | | | | | **7473** |
| 7484 | | | | | |
| | | | | | 7658 |
| | | **7708** | | **7708** | |
| | | | | 7865 | |
| **7869** | **7868** | **7867** | **7868** | **7869** | |
| | | | | **7883** | |
| **8159** | **8158** | | | **8154** | |
| | | 8281 | | 8282 | 8281 |
| | | | **8296** | | |
| | | | | | 8308 |
| | | 8313 | | | |

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
|  | 8326 | 8326 | 8325 | 8327 | 8326 |
|  |  |  |  | 8351 |  |
|  |  |  |  | **8358** | **8355** |
|  |  |  | 8372 |  |  |
|  |  |  | 8395 |  | 8393 |
|  |  | 8422 |  | 8424 |  |
|  | 8433 | 8433 | 8432 | 8434 | 8434 |
|  | 8638 |  |  |  |  |
|  |  |  |  | 8752 | 8750 |
|  |  |  |  | 8761 |  |
|  |  |  |  | 8815 |  |
|  | 8823 | 8822 |  |  |  |
|  | 8848 |  |  |  |  |
|  | 8877 |  |  | 8878 |  |
|  | 8898 | 8898 | 8897 | 8899 | 8897 |
| 9006 | 9005 |  |  |  |  |
|  |  |  |  | 9044 | 9046 |
|  |  | 9063 | 9063 | 9065 | 9063 |
|  | 9120 | 9119 | 9120 | 9119 | 9120 |
| 9191 | 9191 | 9190 | 9190 | 9192 | 9190 |
| 9226 | 9228 |  | 9226 |  | 9225 |
|  |  | 9271 |  | 9273 | 9270 |
|  |  | 9289 |  |  |  |
|  |  |  | 9332 |  |  |
|  | 9339 |  |  |  |  |
|  |  |  |  | 9384 |  |
|  |  | 9535 |  | 9535 | 9536 |
|  | 9555 |  |  |  |  |
|  | 9584 |  |  |  |  |
|  |  |  |  | 9609 |  |
|  | 9684 |  |  |  |  |
|  | 9739 | 9739 | 9738 | 9741 | 9739 |
|  |  |  |  | 9745 |  |
|  | 9753 |  |  | 9757 | 9756 |
|  |  |  |  |  | 9788 |
|  |  | 9826 |  |  |  |
|  |  |  |  | 10107 |  |
|  | 10301 |  |  |  |  |

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| 10315 | 10314 | 10313 |  | 10316 |  |
| 10387 | 10385 |  |  |  |  |
|  |  | 10465 | 10463 | 10466 | 10466 |
|  |  |  |  |  | 10483 |
|  |  |  |  |  | 10497 |
|  |  | 10600 |  | 10605 |  |
|  |  | 10649 |  |  |  |
| 10655 |  |  |  |  |  |
|  | 10692 |  |  |  | 10693 |
|  |  | 10795 | 10795 | 10795 | 10797 |
|  |  |  | 10865 |  |  |
|  |  |  | 11038 |  |  |
|  |  | 11156 |  | 11159 | 11156 |
|  |  |  | 11165 |  | 11170 |
|  |  |  | 11179 |  |  |
| 11186 | 11185 | 11186 |  | 11186 |  |
|  |  |  |  |  | 11222 |
|  |  | 11245 |  |  |  |
|  | 11293 |  |  |  |  |
|  | 11451 |  | 11450 |  |  |
| 11674 | 11675 | 11674 | 11675 | 11674 | 11675 |
|  |  | 11726 |  |  | 11728 |
| 11733 | 11733 | 11735 |  |  |  |
|  |  |  | 11739 | 11737 |  |
|  |  |  | 11893 |  | 11894 |
|  |  | **11977** |  | **11979** |  |
|  |  | 12027 | 12208 | 12028 |  |
|  | 12057 |  |  |  |  |
|  |  |  |  | 12065 |  |
|  |  | 12178 |  | 12183 |  |
|  | **12213** |  |  |  |  |
|  |  | **12219** |  | **12217** | **12219** |
|  |  |  |  | 12524 |  |
|  | 12770 | 12768 | 12769 | 12769 | 12770 |
|  | 12826 |  |  |  |  |
|  | 12861 |  |  |  |  |
|  |  |  |  | 13115 | 13111 |
|  |  |  | 13123 |  |  |

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| | | 13479 | | | |
| | 13482 | | 13482 | 13482 | |
| | | | | | 13524 |
| 13731 | 13734 | | | | |
| | | | 13834 | | |
| | | | | 14083 | 14085 |
| | 14542 | | | | |
| | 14658 | | | | |
| | | | 14864 | | |
| | 14884 | | | | |
| | | 15317 | | | |
| | | 15740 | | | 15742 |
| | | | | 16018 | |
| 16387 | 16385 | 16385 | 16383 | 16390 | |
| | | | | 16660 | |
| | | | | 16862 | |
| | | | | 17056 | |
| | | | | | 17517 |
| | | | 17527 | | |
| | | | | 17542 | |
| | | | | 17573 | |
| 17598 | 17594 | 17591 | 17596 | | 17595 |
| | | 17604 | | 17602 | |
| | | 18079 | | 18076 | |
| | | | 18105 | | 18105 |
| | | 18115 | | 18110 | |
| | | | 18567 | | |
| | | | | 18578 | |
| | | | | | 18595 |
| | | 19477 | | | |
| | | | | 19585 | 19580 |
| | | 20713 | | | |
| | | | | | 20732 |
| | | 21133 | | | |
| | | | 21726 | | |
| | | | | 22262 | |
| | | | 22971 | | |
| | | | | | 22982 |

| 2 h | 4 h | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| | | | | 22991 | |
| | | | | | 24990 |
| | | | | 25025 | |
| | | | | | 25051 |
| | | 27160 | | | |
| | 29346 | | | | |

* Bolded masses represent those whose molecular masses do not match with the proteins in the SWISS-PORT or TrEMBL databases.

**Table A.3** $[M+H]^+$ (Da) from *Pseudomonas fluorescens* (ATCC 948) extracts by LC/off-line MALDI MS.

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 2017 | 2016 | 2016 |
| | | | | | | | 2039 | |
| | | | | | | | 2084 | |
| | | | | | | 2131 | 2130 | 2130 |
| | | | | | | | | 2185 |
| | | | | | | | 2299 | |
| | | | | | | | | 2301 |
| | | | | | 2370 | | | |
| | | | | | | | 2387 | |
| | | | | | | | | 2414 |
| | | | | 2431 | | | | |
| | | | | 2467 | 2467 | | | |
| | | | | | | | 2570 | |
| | | | | | | | 2582 | 2583 |
| | | | | | | | | 2699 |
| | | | | | | | | 3151 |
| | | | 3385 | | | | 3385 | |
| | | | | | | | | 3434 |
| | | | | | | 3542 | 3541 | |
| 3608 | | 3608 | 3608 | 3608 | 3608 | | 3608 | |
| | | | 3629 | 3628 | | | | |
| | | | 4023 | 4023 | | | | |
| | | 4391 | | | | 4389 | 4390 | |
| | | | | | 4409 | 4408 | 4407 | |
| 4436 | 4437 | 4435 | | 4435 | 4436 | 4436 | 4435 | 4436 |
| 4564 | | | | | | | | |
| | | | | | | | 4628 | |
| | | 5063 | | | | | | |
| 5069 | 5069 | | 5070 | 5069 | 5070 | 5070 | 5069 | 5070 |
| | | | | | | | 5085 | |
| | | | 5382 | | | | | |
| | | | 5591 | | | | | |
| 6084 | 6083 | 6083 | 6083 | 6084 | 6083 | 6084 | 6083 | 6083 |
| | | | | | | 6099 | | |
| | | 6203 | 6203 | | | | | |
| | | | | | | 6300 | | |
| | | | 6331 | 6330 | | | | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
| 6396 | 6397 | 6396 | 6396 | 6396 | 6396 | 6398 | 6394 | 6397 |
|  |  |  | 6414 |  |  |  | 6412 |  |
|  |  |  | 6485 |  |  |  |  |  |
| 6613 | 6613 |  | 6614 | 6613 | 6612 | 6614 | 6613 | 6613 |
|  |  | 6748 | 6749 | 6748 | 6749 | 6749 | 6748 |  |
|  |  | 6756 |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 6764 |  |
|  |  |  |  |  |  |  | 6840 | 6839 |
|  |  | 7011 | 7011 |  | 7012 | 7013 |  |  |
| 7160 |  | 7162 | 7162 |  |  |  |  |  |
| 7173 | 7173 | 7173 | 7173 | 7173 | 7173 | 7174 | 7172 | 7173 |
|  |  | 7179 |  |  |  |  |  |  |
|  |  |  |  | 7191 |  |  | 7191 | 7192 |
| 7300 |  | 7300 | 7299 |  |  |  |  |  |
|  |  | 7315 | 7314 | 7314 |  |  |  |  |
|  |  |  |  | 7325 |  |  |  |  |
| 7360 |  | 7361 | 7361 | 7358 | 7359 | 7360 |  | 7360 |
|  |  |  | 7438 | 7442 |  |  |  |  |
|  |  | 7466 |  |  |  |  |  |  |
|  |  |  |  |  | 7497 |  |  |  |
|  | 7542 | 7541 | 7541 | 7543 |  |  |  | 7541 |
| 7564 | 7565 | 7564 | 7564 | 7564 | 7564 | 7565 | 7565 | 7565 |
|  |  | 7571 | 7571 | 7571 |  | 7573 |  | 7570 |
| 7581 | 7582 | 7582 | 7582 | 7581 | 7582 | 7582 | 7583 | 7582 |
|  |  |  |  |  |  |  | 7598 | 7597 |
| 7617 | 7613 | 7615 |  |  |  |  |  | 7613 |
| 7637 | 7638 | 7637 | 7637 | 7637 | 7638 | 7639 | 7637 | 7638 |
|  | 7656 |  |  |  |  |  |  | 7656 |
|  | 7701 | 7702 | 7702 |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 7710 |
| 7849 |  |  |  |  |  |  |  |  |
|  |  |  | 8194 | 8193 |  |  |  |  |
|  |  |  |  |  | 8261 |  |  |  |
| 8328 | 8328 | 8327 | 8328 | 8327 | 8327 | 8326 | 8328 | 8327 |
|  |  | 8406 | 8406 | 8406 | 8407 | 8406 | 8406 |  |
|  |  |  |  | 8443 |  |  |  |  |
|  |  |  |  |  |  |  | 8456 |  |
|  |  |  |  |  | 8616 |  |  |  |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
|  | 8846 | 8842 | 8842 | 8844 |  |  |  |  |
|  |  | 9006 | 9006 | 9007 | 9005 | 9006 |  | 9007 |
|  |  |  |  | 9026 | 9024 |  |  | 9026 |
| 9062 | 9062 | 9061 | 9062 | 9062 | 9062 | 9063 | 9062 | 9062 |
|  |  |  |  |  |  |  | 9074 |  |
|  |  | 9093 | 9093 | 9093 |  |  |  |  |
|  |  | 9115 | 9116 | 9119 | 9119 | 9120 | 9118 | 9120 |
|  |  |  |  |  | 9197 |  |  |  |
|  |  |  | 9209 | 9205 |  | 9210 | 9207 |  |
|  |  | 9233 |  |  |  |  |  |  |
|  |  |  |  | 9324 | 9323 | 9322 | 9321 | 9324 |
|  |  |  |  |  | 9508 |  |  |  |
| 9547 | 9548 | 9547 | 9548 |  |  |  |  | 9548 |
|  |  | 9566 | 9564 | 9565 | 9564 | 9565 | 9565 | 9565 |
|  |  |  |  |  | 9575 |  |  | 9575 |
| 9632 | 9633 | 9632 | 9633 | 9632 | 9632 | 9632 | 9632 | 9633 |
| 9665 |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 9673 |  | 9671 |  |
|  |  | 9706 | 9709 |  |  |  |  |  |
|  |  | 9716 |  |  |  |  |  |  |
|  |  | 9731 |  | 9730 |  |  |  |  |
|  |  | 9756 | 9754 | 9757 |  |  |  |  |
|  |  |  | 9821 | 9819 |  |  |  |  |
| 9837 | 9839 | 9838 | 9839 | 9839 | 9838 |  |  |  |
| 9964 |  | 9963 | 9959 | 9964 | 9963 | 9962 | 9963 |  |
| 10117 |  |  |  |  |  |  |  |  |
| 10128 | 10132 | 10131 | 10130 | 10130 | 10129 | 10130 | 10128 | 10132 |
| 10142 |  | 10146 |  |  | 10145 |  |  |  |
|  |  | 10160 | 10158 | 10165 |  |  |  |  |
|  | 10206 |  |  |  | 10202 |  |  |  |
|  |  |  |  | 10213 |  |  |  |  |
|  |  |  |  |  |  |  | 10315 |  |
| 10342 | 10348 | 10345 | 10345 | 10344 | 10341 |  |  |  |
|  |  | 10413 |  | 10416 | 10414 | 10416 | 10413 |  |
| 10620 |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 10679 |  |  |
|  |  |  |  |  |  |  | 10831 |  |
| 10944 |  | 10942 | 10945 | 10945 | 10943 |  |  | 10946 |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
| | | | | | 10996 | | | |
| | | | | 11143 | 11138 | | | |
| | | | 11156 | | | 11150 | 11148 | |
| | | | | 11160 | | | 11165 | |
| | | | | | 11176 | | | |
| | | 11237 | | | 11235 | | | |
| 11297 | 11299 | 11299 | 11302 | 11300 | 11297 | 11300 | 11297 | 11299 |
| 11414 | | | | | | | | |
| 11699 | | | | | 11693 | | | 11695 |
| | | | | 11905 | | | | |
| | | | 11914 | | | | | |
| | | | | | 11951 | | | |
| | | | | 12343 | 12346 | | | 12345 |
| | | | | 12465 | 12469 | | | |
| | | | 12481 | | | 12476 | 12480 | |
| | | | | | | | 12491 | |
| | | | | | | | 12539 | |
| | | 12720 | | 12716 | | | | |
| | | | | | 12763 | 12764 | 12761 | |
| | | | | | | 12843 | 12840 | |
| | 12903 | 12903 | 12906 | | 12900 | | | |
| 13058 | | 13064 | 13062 | 13062 | 13058 | 13055 | 13060 | |
| | | | | 13222 | | | | |
| | | | | 13398 | 13397 | | | 13394 |
| 13496 | 13497 | | | 13493 | | | | |
| | | | | | 13592 | | | |
| | | | 13622 | 13615 | | | | |
| 13643 | | 13643 | | | | | | |
| | | | | | 14451 | | | |
| 14910 | 14905 | 14911 | | 14906 | 14912 | 14906 | | 14911 |
| | | | 14926 | | | | | |
| | | | 14952 | | | | | |
| | | | 14974 | | | | | |
| 15210 | | | | | | | | |
| | | | 15221 | | | 15225 | 15228 | 15232 |
| | | | | | | 15242 | | |
| | | | | | | 15262 | | |
| | | | 15317 | | | | | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 120 h | 264 h | 432 h |
|---|---|---|---|---|---|---|---|---|
| | | | | 15453 | | | | |
| | | | | | 15466 | 15465 | 15462 | 15465 |
| 15563 | | | 15566 | 15559 | 15567 | 15566 | | |
| | 15578 | | | | | | | |
| | | | | | 15237 | | | |
| 16412 | | 16418 | | 16408 | 16414 | 16419 | | |
| 16424 | | 16427 | 16427 | 16427 | | | | |
| | | 16434 | | | | | | |
| | | | | 16712 | | | | |
| 17587 | | 17589 | | | | | | |
| 17595 | | | 17603 | 17596 | | | | |
| | 17617 | | | | | | | |
| 19255 | | | | | 19245 | | | |
| | | | | | 19737 | | | |
| | | | | | | 20420 | | |
| | | | | | | 20432 | 20430 | |
| | | | | | 20443 | | 20445 | |
| | | | | | | 20455 | 20463 | |
| 20738 | | 20731 | | | 20733 | 20736 | | 20736 |
| | | | 20753 | | | | 20748 | |
| | | | | | 20787 | | | |
| | | 21222 | | | | | | |
| | | | 21235 | | | | | |
| | | 22704 | | | | | | |
| | | | | 26139 | 26147 | | | |
| | | | | 26400 | | | | |
| | 26434 | | 26439 | | | 26437 | | |
| 26450 | | 26444 | | | 26450 | | | 26445 |
| | | 30463 | 30464 | 30462 | | | | |
| | | | | | 30488 | | 30494 | |
| | | | | | | | | 32139 |
| | | | | | | | | 32147 |
| | | | | | | 32180 | | |
| | | | | | 36994 | 37008 | | |
| | | | 37027 | | | | | |

**Table A.4** $[M+H]^+$ (Da) from *Bacillus globigii* (ATCC 9372) extracts by LC/off-line MALDI MS.

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| | | | 2004 | | |
| | | | 2044 | | |
| | | 2079 | | | |
| | | 2092 | | | |
| | | | 2105 | | |
| | | 2118 | | | |
| | 2142 | | | | |
| | | | 2160 | | |
| 2163 | | | | 2163 | |
| | | | | 2169 | |
| | | 2219 | 2220 | | 2220 |
| | | | 2232 | | |
| | | 2247 | | | |
| | 2251 | | | | |
| 2261 | | | | | |
| | 2278 | | | | |
| | | | | 2282 | |
| | | 2317 | | 2318 | 2318 |
| | | | | 2326 | |
| | | | 2332 | | |
| | | | 2348 | | |
| | 2351 | | | | |
| 2365 | | | | 2366 | |
| | 2368 | | | | |
| 2375 | | 2376 | | | |
| 2382 | 2383 | | | | |
| 2394 | 2396 | | | 2395 | |
| 2400 | | 2400 | | | |
| | 2404 | | | | |
| 2407 | | | | | |
| 2410 | | | | | |
| 2423 | 2425 | | | 2424 | |
| | 2430 | | | | |
| | | | | 2439 | |
| | | 2446 | 2447 | | 2448 |
| 2451 | | | | | |
| | | 2454 | | | 2453 |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
|  | 2460 |  |  |  |  |
| 2469 |  |  |  |  |  |
|  | 2494 |  |  | 2495 |  |
|  |  | 2498 |  |  |  |
|  |  |  | 2504 |  |  |
| 2511 | 2510 |  |  |  |  |
|  | 2518 |  |  |  |  |
|  |  |  |  | 2523 |  |
| 2545 |  |  |  |  |  |
|  | 2551 |  |  |  |  |
|  |  | 2563 | 2563 |  | 2563 |
|  | 2565 |  |  |  |  |
| 2573 |  |  |  |  |  |
|  |  |  | 2576 |  |  |
| 2578 |  |  |  |  |  |
| 2582 | 2580 |  |  |  | 2580 |
|  |  |  |  | 2588 |  |
| 2594 | 2595 |  |  | 2594 |  |
| 2597 | 2597 |  |  |  |  |
| 2603 |  |  |  |  |  |
|  |  | 2606 |  |  |  |
|  | 2618 |  |  |  | 2617 |
|  |  |  |  |  | 2625 |
|  |  |  |  | 2629 |  |
|  |  |  |  |  | 2647 |
|  |  |  | 2664 |  | 2665 |
| 2682 | 2682 |  |  | 2682 |  |
| 2685 |  |  |  |  |  |
|  |  |  |  |  | 2690 |
| 2697 | 2699 | 2697 | 2698 | 2696 |  |
|  |  |  |  | 2702 |  |
|  |  |  |  |  | 2704 |
|  | 2708 |  |  |  |  |
|  | 2721 |  |  |  |  |
|  | 2724 |  |  |  |  |
|  | 2728 |  |  |  |  |
| 2730 |  |  |  | 2730 |  |
|  |  |  |  | 2734 |  |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
|  |  | 2743 |  |  |  |
|  |  |  |  | 2754 |  |
| 2761 | 2759 |  |  | 2761 | 2760 |
|  |  |  |  |  | 2770 |
| 2786 |  |  |  |  |  |
|  | 2804 |  |  |  |  |
|  |  |  | 2812 |  |  |
|  |  | 2839 |  |  |  |
|  |  |  | 2845 |  |  |
| 2847 |  |  |  |  | 2847 |
|  |  |  | 2861 |  | 2861 |
| 2863 |  |  |  |  |  |
|  | 2865 |  |  |  |  |
| 2871 |  |  |  |  |  |
|  |  |  |  | 2885 |  |
|  |  |  |  | 2897 |  |
|  |  |  | 2918 |  |  |
| 2923 | 2922 | 2924 |  |  |  |
|  | 2929 |  |  | 2930 |  |
| 2940 | 2941 |  |  | 2939 |  |
|  |  |  |  |  | 2962 |
|  |  |  |  |  | 2971 |
|  |  | 2979 |  |  | 2978 |
| 3003 | 3002 |  |  |  |  |
| 3008 | 3008 | 3008 |  | 3009 |  |
|  | 3022 | 3021 |  |  | 3020 |
|  |  |  |  | 3037 |  |
|  |  |  |  | 3040 |  |
|  |  | 3054 |  |  |  |
| 3066 |  |  |  |  |  |
|  | 3088 |  |  |  |  |
|  | 3092 |  |  | 3091 |  |
|  |  | 3094 | 3095 | 3096 |  |
|  |  |  | 3100 |  |  |
|  |  | 3110 |  |  |  |
|  | 3114 |  |  |  |  |
| 3137 |  |  |  |  |  |
|  | 3150 |  |  |  |  |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| 3155 | | | 3157 | | 3155 |
| | | 3162 | | | |
| | 3166 | | | 3168 | |
| 3177 | 3179 | 3177 | | 3177 | |
| 3185 | | | | | 3187 |
| 3191 | | | | | |
| | 3209 | | | | |
| | 3216 | | 3215 | | |
| | | 3219 | | | |
| 3223 | 3225 | | | | |
| | 3229 | | | | 3230 |
| | | | | | 3245 |
| | 3255 | | | | 3257 |
| | 3262 | | | | 3260 |
| | | | 3271 | | 3269 |
| | | 3276 | | | 3276 |
| | | | | 3281 | |
| 3284 | | | | 3284 | |
| | | | | | 3292 |
| | 3298 | | | | |
| | | 3303 | | | |
| | | | | | 3307 |
| | | | 3313 | | 3315 |
| | 3320 | | | 3323 | 3321 |
| | | 3328 | | | 3329 |
| 3333 | 3332 | | | 3333 | |
| 3338 | | | | 3337 | |
| | 3341 | | | | |
| | | | 3349 | | 3348 |
| | 3352 | | | | |
| | 3356 | | | | 3356 |
| 3383 | 3381 | | | | |
| | | | 3396 | | |
| | | 3418 | 3419 | | |
| 3453 | 3455 | | | | |
| | | | | | 3465 |
| 3476 | 3476 | | | | |
| | | | 3498 | | |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| 3505 | | | | | |
| | | | 3509 | | 3510 |
| | | | | | 3528 |
| 3543 | | | | 3544 | 3544 |
| 3566 | | | 3564 | | 3565 |
| | | | | | 3569 |
| 3575 | | | | 3573 | |
| 3592 | | | | | |
| 3598 | | | | | |
| 3610 | | | | | |
| 3626 | | | | 3627 | 3629 |
| 3638 | | | | | |
| | | | | | 3645 |
| 3678 | 3678 | | | | |
| | | | | | 3681 |
| | 3693 | | | | |
| | | | 3700 | 3701 | |
| | 3711 | | | | 3713 |
| | 3715 | 3717 | 3715 | | |
| | | 3727 | | | |
| | | | | 3730 | 3730 |
| 3735 | | | 3733 | | |
| | 3744 | | | | |
| | | | 3747 | | 3747 |
| | | | 3773 | | |
| | | | 3784 | | |
| | 3797 | | | | |
| | | | 3816 | | |
| 3829 | | | | | |
| 3833 | | | 3833 | | |
| | | | 3838 | | |
| | | 3842 | | 3844 | |
| | | 3858 | | | 3857 |
| | | 3871 | 3869 | | |
| | | 3892 | 3893 | | 3894 |
| 3900 | | 3899 | | 3901 | |
| | | | 3910 | | |
| | | | 3918 | | |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| | | | 3930 | | |
| | | 3934 | 3934 | | |
| | | 3946 | | | 3947 |
| | | 3949 | 3949 | | |
| | | | 3955 | | |
| | | | 3968 | | |
| | | 3974 | | | |
| | | 3991 | | | |
| | 4001 | | | | |
| | | | 4009 | 4008 | 4009 |
| | | | 4019 | | 4017 |
| 4022 | 4020 | | | | |
| | | | 4026 | | |
| 4040 | | | 4040 | | |
| | | | | | 4069 |
| 4080 | | | | | |
| 4087 | | | | | |
| 4091 | | | | | |
| | 4100 | | | 4099 | |
| 4104 | | | | | |
| 4114 | 4116 | 4116 | | 4115 | |
| | | | | 4131 | |
| | | | | 4142 | |
| | 4154 | | | | |
| 4169 | | | | | |
| | | | | | 4182 |
| | | 4195 | 4196 | | 4197 |
| | | 4205 | | | |
| | | 4209 | | | |
| | | | | 4229 | |
| | | | 4250 | 4249 | |
| 4262 | | | | | |
| | | | 4266 | | |
| 4271 | | | | 4270 | |
| 4286 | 4284 | | | | |
| | | 4291 | 4290 | 4292 | |
| | | 4298 | | | 4296 |
| | | | 4305 | | |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| | | 4311 | 4312 | | 4312 |
| | | | | 4315 | |
| | | 4322 | | | |
| 4334 | | | 4336 | 4337 | |
| | | 4403 | 4405 | | |
| | | | | | 4412 |
| | | | 4432 | | |
| | | | 4456 | | |
| | 4496 | | | | |
| 4507 | | | | | |
| | | | | 4514 | |
| 4524 | 4524 | | 4523 | | |
| 4534 | | | | | |
| | | | | 4547 | |
| | | | | 4560 | |
| | | | 4606 | | |
| | | | 4622 | | |
| | 4664 | | | | |
| | 4679 | 4681 | 4680 | 4681 | |
| | | | | | 4711 |
| 4751 | 4748 | | | | |
| 4782 | | | | | |
| | | 4793 | 4792 | | |
| 4882 | 4881 | | 4882 | | |
| | | 4899 | | | |
| | | 4943 | 4944 | | |
| | | | 4964 | | |
| | | | 4981 | 4982 | |
| 4994 | | | | | |
| | | | 4997 | | |
| 5002 | | | | | |
| 5032 | | | | | |
| 5066 | 5068 | | | | |
| 5073 | | 5071 | | | |
| 5099 | 5097 | | | | |
| 5119 | | | | | |
| 5147 | | | | | |
| | | 5206 | 5208 | | |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
|  | 5213 |  |  |  |  |
|  |  | 5223 | 5224 |  |  |
|  |  |  |  |  | 5236 |
| 5289 |  | 5291 |  |  |  |
|  |  |  |  | 5326 |  |
|  |  | 5350 | 5350 |  |  |
|  |  |  | 5360 |  |  |
|  | 5400 |  |  |  |  |
|  |  | 5406 |  |  |  |
|  |  | 5441 |  |  |  |
| 5526 |  |  |  |  |  |
|  |  | 5620 |  |  |  |
|  |  | 5634 | 5636 |  | 5635 |
|  |  |  | 5647 |  |  |
|  |  | 5661 |  |  |  |
|  |  |  |  |  | 5669 |
|  | 5698 |  |  |  |  |
| 5712 | 5712 |  |  |  |  |
|  |  | 5746 | 5747 |  |  |
| 5812 |  |  |  |  |  |
|  |  |  | 5821 |  |  |
|  |  |  | 5835 |  |  |
|  |  | 5847 | 5850 |  |  |
|  |  |  | 5874 | 5875 |  |
|  |  | 5980 |  |  |  |
|  |  |  |  | 6012 |  |
|  | 6061 | 6064 | 6063 | 6064 |  |
|  |  | 6160 | 6162 |  |  |
|  |  |  |  | 6215 |  |
| 6236 |  |  |  |  |  |
|  |  | 6539 | 6537 |  |  |
|  |  |  | 6543 |  |  |
|  |  |  |  | 6564 |  |
|  | 6640 |  |  |  |  |
|  |  | 6867 | 6870 | 6868 |  |
|  | 6996 |  |  |  |  |
|  | 7162 |  |  |  |  |
|  | 7237 |  | 7236 |  |  |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
|  | 7250 | 7251 |  |  |  |
|  | 7270 | 7269 | 7268 | 7268 | 7266 |
|  |  |  |  | 7280 |  |
| 7296 | 7293 | 7294 | 7296 | 7297 | 7295 |
|  |  |  | 7302 |  |  |
|  | 7307 | 7306 |  |  |  |
| 7319 | 7319 |  |  |  |  |
| 7326 | 7326 | 7326 | 7326 | 7325 | 7324 |
| 7348 | 7352 | 7353 | 7353 | 7353 | 7353 |
|  | 7397 |  |  |  |  |
|  |  |  | 7723 |  |  |
|  |  |  | 7728 |  |  |
|  |  |  |  | 8062 |  |
|  |  |  |  | 8079 |  |
|  |  |  |  | 8106 |  |
|  |  |  |  | 8220 |  |
|  |  |  |  | 8491 |  |
|  |  |  |  | 8497 |  |
|  |  | 8502 |  |  |  |
|  |  |  |  | 8595 |  |
|  |  |  |  | 8631 |  |
|  | 8646 |  |  | 8649 |  |
|  | 8724 |  |  |  |  |
|  |  |  |  | 8757 |  |
|  | 8772 |  |  |  |  |
|  |  |  |  |  | 8846 |
|  |  |  |  |  | 8898 |
|  |  |  |  |  | 8901 |
|  |  |  |  |  | 8914 |
|  |  |  |  |  | 8934 |
|  |  |  |  | 9192 |  |
|  |  | 9200 |  |  |  |
|  |  |  |  | 9322 |  |
|  | 9381 |  |  |  |  |
|  |  |  | 9763 | 9768 |  |
|  |  | 9777 |  | 9775 |  |
| 9831 |  |  |  |  |  |
|  | 9888 |  |  |  |  |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| 9895 | 9893 | 9892 | 9897 | | |
| 9905 | | | | | |
| | | 9924 | | | |
| | | 9975 | | | |
| 9995 | | | | | |
| | | 10004 | | | |
| 10059 | | | | | |
| 10068 | | | | | |
| | 10099 | | | | |
| | 10105 | | | | |
| 10119 | 10119 | | | | |
| | | 10308 | | | |
| | | | | 10322 | |
| | 10339 | | | | |
| | 10345 | 10349 | | | |
| | 10505 | | | | |
| 10819 | | | | | |
| 10926 | | | 10927 | | |
| 11013 | | | | | |
| | 11167 | | | 11170 | |
| | | | 11275 | | |
| | | | | 11297 | |
| | 11309 | | 11304 | 11304 | 11307 |
| | | 11296 | | | |
| | 11323 | | | | 11322 |
| | 11348 | | | | |
| | 11360 | | | | 11359 |
| | | | 11574 | | |
| | 11582 | 11587 | 11584 | 11586 | |
| | 11590 | 11593 | 11595 | 11599 | |
| | | 11615 | | | |
| | | 12003 | | | 12006 |
| | | | | | 12047 |
| | | 12382 | | | |
| | 12409 | | | | |
| | | | | 12700 | |
| | | | | 15823 | |
| | | 17199 | 17192 | | |

| 11h | 16h | 24h | 36h | 48h | 72h |
|---|---|---|---|---|---|
| | | | | 17207 | |
| | 17283 | 17280 | | 17272 | |
| | | | | 17504 | |
| | | 19538 | | 19544 | |
| | | 19563 | | | |
| | | 20625 | | | |
| | | 20648 | | | |
| | | | | 20666 | |
| | 20677 | | | | |
| | 20702 | | | | |
| | 21008 | | | | |

**Table A.5** [M+H]$^+$ (Da) from *Bacillus subtilis* (ATCC 23857) extracts by LC/off-line MALDI MS$^*$.

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| | | | | | | **2461** | |
| | | | | | | | **2872** |
| | | | | | | | **3048** |
| | | | | | | **3180** | |
| | | | | | | **3299** | |
| | | | | | | **3325** | |
| | | | | | **3445** | | |
| | | | | | **3464** | **3465** | |
| | | | | | | **3483** | |
| | | **3549** | | | | | |
| | | **3558** | | | | | |
| | | | | | | | **3638** |
| | | | | | | **3641** | |
| | | | | | | **3701** | |
| | | | | | 3714 | **3715** | |
| | | | | | | **3735** | **3735** |
| | | | | | **3752** | **3750** | |
| | | | | | **3755** | **3754** | |
| | | | | | **3773** | | |
| | | | | | **3846** | | |
| | | | | | **3883** | | |
| | | | | | **3956** | | |
| | | | | | | **4010** | |
| | | | | | | | **4020** |
| | | | | | | | **4035** |
| | | | | | | **4048** | |
| | | | | | | **4102** | |
| 4173 | 4173 | | | | | | |
| | | | | | | **4179** | |
| | | | | **4255** | | **4255** | **4254** |
| | | | | | 4277 | 4276 | 4275 |
| | | | | | | | **4280** |
| | **4295** | | | | | | **4297** |
| | | | | | **4304** | | |
| | | | | 4311 | 4311 | 4312 | |
| | | | | | **4364** | | |
| | | **4375** | | | | | |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
|  |  |  |  |  | 4381 |  |  |
| 4408 | 4409 |  |  |  |  |  |  |
|  |  |  |  |  | 4417 |  |  |
|  |  |  |  | 4423 |  |  |  |
|  | 4428 | 4426 |  |  |  |  |  |
|  |  |  |  |  | 4435 |  |  |
|  |  |  |  | 4439 | 4438 | 4440 |  |
|  |  |  |  |  | 4464 |  |  |
|  |  |  |  |  | 4531 |  |  |
| 4541 |  |  |  |  |  |  |  |
| 4551 |  |  |  |  | 4552 | 4552 | 4553 |
|  |  |  |  | 4580 | 4581 | 4583 |  |
|  |  |  |  |  | 4693 |  |  |
|  |  |  |  |  | 4704 |  |  |
|  |  | 4735 |  |  |  |  |  |
|  |  |  |  |  | 4782 |  |  |
|  |  |  |  |  | 4805 | 4806 |  |
|  |  |  |  |  |  | 4820 |  |
|  |  |  |  | 4881 | 4882 | 4882 |  |
|  |  |  |  |  | 4906 |  |  |
|  |  | 5097 | 5097 |  |  |  |  |
|  |  |  |  |  |  | 5112 | 5111 |
|  |  |  |  | 5123 |  | 5124 |  |
|  |  | 5228 |  |  |  |  |  |
|  | 5255 |  |  |  |  |  |  |
|  | 5275 | 5274 |  |  |  |  |  |
|  |  |  |  |  |  | 5283 |  |
|  | 5351 |  |  |  |  |  |  |
|  | 5381 |  |  |  |  | 5383 | 5382 |
|  |  | 5510 |  |  |  |  |  |
|  |  | 5568 |  |  |  |  |  |
|  |  |  |  |  | 5663 | 5663 |  |
|  |  |  |  |  | 5671 |  |  |
|  | 5680 | 5679 |  |  |  |  |  |
|  |  |  |  |  |  | 5689 |  |
|  |  |  |  |  | 5731 |  |  |
|  |  |  |  |  |  | 5818 | 5816 |
|  |  |  |  | 5901 | 5901 |  |  |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| | | | | 5913 | 5914 | | |
| | | | | | **5948** | | |
| | | | | 6064 | | | |
| **6072** | | | | | | | |
| | | | **6094** | **6093** | | **6094** | **6095** |
| | | | | | | | **6153** |
| | | | 6259 | | | | |
| | | | | | | | **6350** |
| **6383** | **6382** | | | | | | |
| | | | | | | | 6404 |
| | | | | **6498** | | | |
| 6507 | 6510 | | | | | | 6508 |
| | | | | | | | **6534** |
| | | | | | | **6552** | **6551** |
| 6597 | 6597 | | | | | | |
| | | | | 6648 | | | |
| | 6699 | | | | | | |
| | | | | **6711** | | | **6707** |
| | | **6761** | | | | **6764** | |
| | | | | | | **6830** | **6830** |
| | | | | | | **6850** | **6851** |
| | | | **6873** | | | | |
| | | | | 6880 | | | |
| | | | | | | **6885** | **6886** |
| | | | | | | | 6924 |
| | | | | | | **6941** | **6941** |
| | | | | | | **6955** | |
| 7112 | 7112 | | | | | | |
| 7256 | 7255 | 7255 | | 7255 | 7255 | 7257 | 7255 |
| | | | | | | 7270 | |
| 7274 | 7277 | | | | | | |
| **7283** | **7283** | **7282** | | | | | |
| | | 7294 | | | | | |
| **7303** | **7301** | **7304** | | | | | |
| | **7313** | **7314** | | **7309** | **7309** | **7310** | **7310** |
| 7337 | 7339 | 7338 | 7337 | 7337 | 7337 | 7337 | 7337 |
| | | | | | 7342 | | |
| **7351** | | | | | | | |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| 7364 | 7366 | 7366 | 7366 | 7365 | 7365 | 7365 | 7366 |
| | | **7374** | | | | | **7376** |
| | 7385 | | | | | | 7382 |
| **7393** | **7393** | **7393** | **7393** | **7393** | **7393** | **7393** | **7392** |
| | | | **7399** | | | **7397** | |
| 7410 | | 7409 | 7411 | | 7411 | 7408 | |
| | | | **7417** | | | | |
| | | | | | 7423 | | |
| | 7427 | | | | | | |
| 7437 | | | | | | | |
| 7440 | 7441 | 7442 | | | | | |
| | | | | | | **7447** | |
| **7452** | | | **7454** | **7455** | **7455** | **7451** | |
| 7462 | | | | | | | |
| | | | | | | | **7529** |
| 7673 | 7669 | | | | | | |
| | **7691** | | | | | | |
| 7695 | 7699 | | | | | | |
| 7711 | 7713 | 7714 | 7714 | | | 7715 | 7714 |
| | | | | | | | **7730** |
| **7738** | **7741** | **7741** | | | **7741** | | |
| | | | | | | | 7756 |
| **7766** | | | | | | | |
| | **7771** | **7771** | **7775** | | **7773** | | **7774** |
| 7793 | 7798 | 7797 | | | | | |
| **7808** | | **7812** | | | | | |
| **7835** | | | | | | | |
| 7844 | | | | | | | |
| 7854 | | | | | | | |
| | | | | | | | 7867 |
| **8007** | | | | | | | |
| | | **8032** | | | | | |
| 8084 | | | | | | | |
| | | | | | | | **8198** |
| | | 8249 | | | | 8253 | 8252 |
| 8337 | | | | | | | |
| | 8342 | | | | | | |
| | | | | | | **8358** | **8360** |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| | | | | 8459 | | | |
| | | | | 8513 | | 8512 | 8515 |
| | | | | 8541 | | 8542 | |
| | | | | | | **8571** | |
| | | | | 8588 | | | |
| | | | | | | 8735 | |
| | | | | | | | **8747** |
| | | | | | | | 8798 |
| 8837 | 8839 | 8838 | | | | | |
| | **8919** | **8919** | | | | | |
| 9057 | 9057 | 9058 | | 9059 | | | |
| | | | 9120 | | | | 9116 |
| | | **9187** | | **9190** | | **9189** | |
| 9207 | 9208 | | | | | | 9210 |
| | | | | 9338 | | | |
| | | | | 9347 | | 9346 | |
| **9509** | | **9508** | | | | | |
| | | | | | | | 9516 |
| | | **9580** | | | | | |
| | | | 9800 | 9799 | 9799 | 9798 | 9800 |
| | | 9855 | 9860 | | | | |
| 9883 | 9882 | 9885 | 9884 | 9883 | 9881 | 9884 | 9884 |
| 9911 | 9910 | 9913 | 9913 | 9912 | 9911 | 9908 | |
| | **9938** | | | | | | |
| | | | 9947 | | | | |
| | **9964** | **9969** | | | | | |
| 10004 | 10005 | 10005 | | | | | 10004 |
| | | 10068 | | | | | |
| **10097** | | | | | | | |
| | | | | | | | **10130** |
| | | | 10175 | 10176 | | 10176 | 10177 |
| | | 10186 | | | | | |
| | | 10202 | | | | | |
| 10442 | 10441 | 10440 | 10442 | | | | |
| 10449 | 10454 | | | | | | 10454 |
| | | | | | | | **10481** |
| | | 10498 | | | | | |
| 10863 | | 10860 | 10861 | 10860 | | 10861 | 10859 |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| **10883** | | | | | | | |
| | | | | 10948 | | | 10950 |
| | | | | | | **11123** | **11125** |
| 11140 | | 11144 | 11141 | | | | |
| | | | | | | | 11167 |
| **11180** | | | | | | **11178** | |
| | | 11220 | 11214 | 11218 | | 11214 | |
| 11262 | | 11260 | | 11259 | | 11259 | 11261 |
| 11275 | | 11277 | | | | | |
| | | | | | | | **11286** |
| | | | **11343** | | | | |
| **11531** | | | | | | | |
| | | | | | | 11592 | 11596 |
| | | | | 11665 | | | |
| | | 11973 | | | | | |
| | | | | 12002 | | | |
| | | | | | | 12111 | 12115 |
| | | | | | | | **12270** |
| | | | 12425 | | | | 12430 |
| 12457 | 12457 | 12459 | | | | | |
| 12466 | | | | | | | |
| | 12618 | 12620 | 12619 | 12619 | 12617 | 12616 | 12618 |
| | | | | | **12667** | | |
| | | 12745 | 12745 | | 12746 | 12746 | 12746 |
| | | | 12750 | | 12752 | | |
| 12969 | 12968 | | 12969 | | | | |
| | | | | | **13411** | **13405** | **13412** |
| | | 13462 | 13458 | 13460 | 13460 | 13461 | 13458 |
| | | | 13623 | | | | 13618 |
| 13653 | 13650 | 13656 | 13652 | | | | |
| | | | | | | | 13664 |
| | | **13752** | | **13747** | | | |
| | | | | **13858** | | | |
| 14182 | 14176 | 14179 | | | | 14177 | 14176 |
| | | | | | | **14207** | |
| **14217** | | **14220** | | | | | **14215** |
| | | | | **14304** | | | |
| | | | | | | | **14313** |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| 14891 | | | | | | | |
| **14923** | | | | | | | |
| | | **15146** | | | | | |
| 15263 | 15257 | | | | | | 15258 |
| | | | | | | 15271 | |
| 15387 | | 15387 | | | | | |
| | | | **15455** | **15450** | **15447** | **15456** | |
| | | | | | | | **15513** |
| | | | 16203 | | | | |
| | | 16218 | | | | | |
| 16298 | | | | | | | |
| | | | | | 16608 | | |
| | | | | | 16635 | | |
| | | | | | | 16759 | 16758 |
| | | | **16818** | **16817** | **16818** | | |
| | | **16823** | | **16823** | | | |
| | | | | | **16836** | | |
| | | | **16963** | **16959** | **16958** | **16952** | **16959** |
| | | | | **16979** | | | |
| 17141 | | | | | | | |
| | | | | 17231 | | | |
| | | | 17332 | 17334 | 17339 | | |
| | | | | | | **17453** | |
| | | | 17621 | | | | |
| | | | | | **17689** | | |
| | | | 17750 | | | | |
| | | 17762 | | | | | |
| | | 18083 | | 18085 | 18078 | 18084 | 18078 |
| | | | 18095 | | | | |
| | | | **18206** | | | | |
| | | | | | **18231** | | |
| | | | | 18242 | | | |
| | | | | | 19680 | | |
| | | | | | | | 20469 |
| | | | | | | | 20495 |
| | | | | | | | **20549** |
| | | | | | | | 20564 |
| | | | 20626 | | | | 20620 |

| 12 h | 14 h | 18 h | 24 h | 48 h | 72 h | 144 h | 250 h |
|---|---|---|---|---|---|---|---|
| | | 20635 | | 20637 | | | |
| | | | | | | 21538 | |
| | | | | | | **22352** | **22355** |
| | | 22362 | | 22368 | | | |
| | | **22372** | | | | | |
| | | 27030 | | | | | |
| | | | 27315 | | | | |
| | | | | 27340 | | | |

* Bolded masses represent those whose molecular masses do not match with the proteins in the SWISS-PORT or TrEMBL databases.

**Table A.6** [M+H]$^+$ (Da) from *Bacillus megaterium* (ATCC 14581) extracts by LC/off-line MALDI MS.

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | | | 2002 |
| 2054 | | | | | | | |
| | | | | | | | 2148 |
| | 2282 | 2283 | | | | | 2283 |
| | | | | | | | 2329 |
| | | 2371 | | | | | |
| | | 2396 | | | | | |
| | | | | | | | 2401 |
| 2410 | | | | | | | |
| | | | | | | | 2413 |
| | 2417 | | | | | | 2417 |
| 2424 | | | | | | | |
| | 2428 | | | | | | |
| | | 2439 | | | | | |
| | | 2446 | | | | | |
| 2465 | | | 2465 | | | | |
| | 2474 | | | | 2473 | 2474 | |
| | | 2478 | | | | | 2478 |
| | 2549 | 2550 | | | | | |
| 2565 | | | | | | | |
| | | | 2584 | | | | |
| | | 2588 | 2588 | 2588 | | | |
| | | | | | | | 2619 |
| | | | | | | | 2622 |
| | | | 2628 | 2629 | | | |
| | | | | | | 2638 | |
| | 2669 | | | | | 2670 | 2668 |
| | | 2689 | | | | | |
| | | | 2704 | 2703 | | | |
| | 2718 | 2717 | | 2719 | | | 2718 |
| | | | 2730 | 2729 | 2730 | | |
| | 2740 | 2740 | 2741 | 2740 | 2741 | 2741 | |
| | | | 2757 | 2756 | | | |
| | | | | | | | 2770 |
| 2775 | | | | | | | |
| 2782 | | | | | | | |
| | 2827 | | | | | | |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 2840 | | | | | | | |
| | 2871 | 2870 | | | | | |
| 2877 | | | | | | | 2894 |
| | | | 2906 | 2906 | 2907 | | |
| | | 2909 | | | | | |
| | | | 2923 | | | | |
| 2933 | 2933 | 2933 | | | | 2933 | |
| 2953 | 2952 | 2952 | | | | | |
| | | | | | | | 2963 |
| | | | 2970 | 2970 | | | |
| | | | | | | 2981 | |
| 3004 | | | | | | | |
| | | 3015 | | | | | |
| | | | 3021 | 3021 | | | |
| | | | 3026 | 3025 | | | |
| | 3045 | 3046 | 3046 | 3047 | | | |
| 3067 | 3067 | | | | | | |
| | | | 3075 | 3075 | | | |
| | | | | | 3084 | | |
| | | | | | | | 3090 |
| | | | | | 3094 | | |
| | | | 3110 | | | | 3098 |
| 3119 | | | | | 3119 | 3118 | |
| | | 3121 | | | | | 3122 |
| | | 3135 | | | | | 3135 |
| | | | | | | | 3150 |
| | 3153 | | | | | | |
| | 3192 | 3193 | 3193 | | | | 3193 |
| | 3208 | 3206 | | 3207 | 3208 | 3208 | 3208 |
| | | | 3212 | 3212 | 3212 | 3212 | |
| | | | | | | | 3218 |
| | | | | | 3252 | | |
| 3265 | | | | | | | |
| | | | 3276 | 3275 | 3276 | 3277 | |
| | | | | | | 3285 | 3286 |
| | 3289 | | | | | | |
| | | | 3321 | | | | |
| | | | 3334 | | | | |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | 3338 | | | |
| | 3367 | 3364 | | | 3367 | 3366 | 3366 |
| | | 3390 | | | | | |
| | 3420 | 3421 | | | | | 3421 |
| | | | 3432 | | | | |
| | | | 3450 | 3449 | | | |
| | | 3463 | | | | | 3463 |
| | | | | 3475 | | | |
| 3495 | | | | | | | |
| | | | | | | | 3506 |
| | | 3516 | | | | | |
| | | | 3536 | 3536 | | | |
| | 3562 | | 3563 | | 3563 | | |
| | | 3573 | | | | | |
| | | 3589 | | 3590 | | | |
| | | | 3596 | | 3597 | | |
| | | 3622 | | | | | |
| | | | | | | | 3631 |
| | | | 3668 | 3669 | | 3668 | |
| | | | | | 3732 | | |
| | | | 3737 | | | | |
| | | 3749 | | 3748 | 3750 | 3749 | 3747 |
| | | | 3755 | 3754 | | 3756 | |
| | 3775 | 3777 | | | | | |
| | | 3797 | | | | | |
| | | | | | | | 3805 |
| | | | | | 3830 | | |
| 3833 | | | | | | | |
| | | | 3842 | 3841 | 3842 | 3843 | |
| | | | | 3853 | 3853 | | |
| | | | 3858 | | | | 3859 |
| | | | | | | | 3869 |
| | | | | | | | 3875 |
| | | | 3882 | | | | |
| | | 3895 | | | | | |
| | | | | | | | 3904 |
| | 3908 | | | | | | |
| | | | | | | | 3926 |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | 3935 | | | | |
| | | | 3944 | | | | |
| | | 3947 | | | | | |
| | 3951 | 3952 | | | | | |
| | 3970 | | | | 3971 | | |
| 3995 | | | | | | | |
| | 4009 | 4009 | | | | | |
| | | | 4067 | | | | |
| | 4070 | | | | | | |
| | | 4117 | | | 4117 | | |
| | 4131 | | 4132 | 4132 | | | 4129 |
| 4141 | | | 4143 | 4141 | | | |
| | | | | | 4146 | | |
| | | | | 4166 | | | |
| | | 4173 | | | | | 4173 |
| | | | | | | | 4193 |
| | | | | | | | 4205 |
| | | 4234 | | | | | 4233 |
| | 4237 | | | | | | |
| | | | | | 4242 | 4242 | |
| | | | | | | | 4296 |
| | | | | | | | 4306 |
| | | | | | | 4316 | |
| | | | | | | 4336 | |
| | | | | | 4367 | | |
| | | 4389 | | | | | 4390 |
| | | | | | | | 4398 |
| | | | 4421 | 4422 | | 4424 | |
| | | 4452 | 4447 | 4447 | | 4449 | |
| | 4469 | 4470 | | | | | 4470 |
| | | | 4513 | | | | |
| | 4518 | | | | | | |
| | | 4560 | | | | | |
| | | | 4567 | | | | |
| | 4574 | 4575 | 4575 | | | 4575 | |
| | | | 4583 | | | | |
| | | | | | | 4607 | 4606 |
| | | | | | | | 4626 |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | 4649 | 4649 | | | |
| | | | 4656 | 4656 | | | 4656 |
| 4724 | | | | | | | |
| | 4733 | 4733 | | | | | 4732 |
| | | | 4750 | 4750 | | | |
| | | | 4754 | | 4754 | | |
| | | | 4763 | | | | 4762 |
| | 4776 | 4776 | | | | | |
| | | | 4787 | | 4785 | | |
| | | 4791 | 4792 | | | | |
| | | | | | | 4803 | |
| | | | | 4842 | | | |
| | 4868 | 4868 | | 4869 | 4870 | | 4868 |
| | | | 4902 | | | | |
| | | | | | 4909 | | 4910 |
| | 4914 | 4914 | | | | | |
| | | | 4923 | 4923 | | 4924 | |
| | | | | | 4954 | | |
| | | 4971 | 4971 | 4970 | 4971 | 4971 | |
| | | | 5081 | | | | |
| | | | | | 5109 | | |
| | 5115 | | 5114 | | 5113 | 5112 | |
| | 5132 | | | | | | |
| | | | | | 5138 | 5140 | |
| | | | | 5143 | | | |
| | 5187 | | | | | | |
| | | | 5192 | 5192 | | | |
| | 5207 | 5208 | | | 5208 | | 5208 |
| 5216 | | | | | | | |
| | 5229 | 5230 | 5229 | | 5229 | 5229 | 5229 |
| | | | 5249 | 5249 | | | |
| | 5296 | | | 5297 | 5299 | 5298 | 5297 |
| | 5334 | | | | | | |
| | | | | | | 5397 | 5399 |
| | | | | | | | 5414 |
| | | | | | | | 5443 |
| | 5510 | 5509 | | 5509 | | 5511 | 5511 |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | 5518 | | | | | |
| | | | | | | | 5562 |
| | 5613 | | | | | 5614 | |
| 5650 | | 5649 | | | | | |
| | | | 5659 | | | | |
| | 5698 | 5699 | | | | | |
| | 5714 | | | | | | |
| | | | | | | 5769 | |
| | | | 5784 | | | | |
| | | 5817 | 5817 | 5818 | 5822 | 5819 | |
| 5832 | 5832 | 5831 | | | | | |
| | | | 5836 | | | | |
| | | | | | | 5910 | |
| | 5933 | | | | | | |
| | | | 5951 | | | | |
| | | | | | | 6038 | |
| | | | | | | | 6042 |
| | | 6099 | | | | | |
| | | | | | | | 6118 |
| | | | 6133 | | | | |
| | 6140 | 6138 | | | | 6140 | 6140 |
| 6164 | | | | | | | |
| | | | | | 6229 | | |
| | | | | | 6251 | | |
| | | | 6255 | | | | 6257 |
| 6291 | | | | | | | |
| 6363 | | | | | | | |
| | 6371 | 6368 | 6368 | | | | |
| | | | | | 6379 | | |
| 6396 | 6399 | | | | | | |
| 6432 | | | | | | | |
| | | | | 6438 | | | |
| | | | 6455 | | | | |
| | | | | 6533 | | | |
| 6539 | 6538 | 6537 | 6538 | 6538 | 6538 | 6538 | |
| | 6543 | | | | | | |
| 6577 | 6576 | | | | | | |
| | | | | 6589 | | | |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | 6683 | | 6682 | | | | |
| 6708 | 6708 | | | | | | |
| 6745 | 6745 | 6744 | | | | | |
| | 6852 | 6852 | | | | 6854 | 6853 |
| | | | | | | 6896 | |
| | | | 6918 | | | | |
| | 6964 | | | | | | |
| | | | | | | 6995 | |
| | | | | 7028 | | 7029 | |
| | | | 7061 | 7062 | 7061 | 7061 | 7061 |
| | | | | | | | 7096 |
| | | | | | | 7125 | |
| | | | | | | | 7130 |
| | | | | | | 7149 | |
| 7167 | 7168 | 7168 | 7168 | | | | |
| | | | | | | | 7173 |
| | 7192 | 7193 | 7193 | 7193 | 7192 | | 7193 |
| 7205 | 7206 | | 7207 | 7206 | | | |
| | 7219 | 7220 | | | | | |
| | | 7234 | | | | | |
| | 7251 | 7250 | | | 7248 | 7254 | 7250 |
| | | 7257 | 7258 | | 7258 | 7259 | |
| | 7278 | 7277 | 7277 | 7276 | 7276 | 7277 | 7276 |
| 7280 | | 7280 | 7280 | 7279 | 7280 | 7280 | 7280 |
| | | 7285 | | | | | |
| 7307 | 7308 | 7308 | 7309 | 7310 | 7311 | 7308 | 7311 |
| 7320 | 7318 | 7319 | 7318 | 7319 | 7319 | 7319 | 7319 |
| 7339 | 7338 | 7338 | 7337 | 7340 | 7342 | 7342 | 7341 |
| | | | 7345 | | 7348 | 7348 | 7349 |
| 7354 | 7351 | 7351 | 7353 | 7352 | 7354 | 7353 | |
| | | | 7373 | | 7372 | | 7372 |
| | 7377 | 7376 | 7376 | 7375 | 7377 | 7375 | |
| 7382 | | | | | | | |
| | | | 7389 | 7390 | | | 7390 |
| | | | | 7399 | 7398 | 7398 | |
| | | | | | | 7407 | |
| | | | 7416 | 7417 | 7417 | 7417 | |
| | 7426 | 7428 | 7427 | 7427 | 7427 | | |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 7450 | 7450 | 7449 | 7450 | 7450 | 7451 | 7450 | 7450 |
|  |  |  | 7459 | 7460 |  |  |  |
| 7478 | 7478 | 7477 | 7477 |  |  |  |  |
| 7495 | 7492 | 7490 |  |  |  |  |  |
|  |  |  | 7513 |  |  |  |  |
|  | 7580 | 7578 |  |  |  | 7578 |  |
|  |  |  | 7600 |  |  |  |  |
|  |  |  |  |  | 7703 |  |  |
| 7708 |  |  |  |  |  |  |  |
|  |  |  |  |  | 7721 | 7721 | 7721 |
| 7729 | 7730 | 7728 | 7729 |  |  | 7729 | 7729 |
|  |  |  |  | 7742 |  |  |  |
| 7758 | 7758 | 7756 |  |  |  |  |  |
|  |  |  |  | 7781 |  |  |  |
|  | 7870 | 7868 |  |  |  |  | 7869 |
|  |  | 7924 |  |  |  |  |  |
|  |  |  |  |  |  |  | 8018 |
|  |  |  |  | 8049 |  |  |  |
|  | 8107 | 8105 |  |  |  |  | 8107 |
|  |  | 8143 |  |  |  |  |  |
| 8213 |  |  |  |  |  |  |  |
| 8225 |  |  |  |  |  |  |  |
|  | 8239 | 8241 |  |  |  |  |  |
|  |  |  | 8261 |  |  |  |  |
| 8272 |  |  |  |  |  |  |  |
|  |  | 8306 |  |  |  |  | 8308 |
| 8328 |  | 8324 |  |  |  |  |  |
|  |  | 8352 |  |  |  |  |  |
|  |  | 8383 |  |  |  |  | 8384 |
|  |  | 8400 |  |  |  |  |  |
|  |  |  |  |  |  |  | 8429 |
|  |  |  |  | 8476 |  |  |  |
|  |  |  |  |  | 8614 |  |  |
|  |  |  |  | 8618 | 8618 |  |  |
|  |  |  |  | 8663 |  |  | 8663 |
|  |  | 8797 |  |  |  |  |  |
|  | 8801 |  | 8799 |  | 8799 |  |  |
|  |  | 8824 | 8820 |  |  |  |  |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | 8850 | | | | | |
| | | | | | | 8921 | |
| | 8933 | | 8930 | | | | |
| | | 8957 | | | | | |
| | | | | 8965 | | | |
| | | 8989 | 8988 | 8988 | 8989 | 8989 | 8989 |
| | 9053 | 9052 | 9053 | 9054 | | 9054 | |
| | | | 9080 | 9081 | | | |
| | 9120 | 9120 | 9119 | 9120 | 9120 | 9121 | |
| | 9305 | | | | | | |
| 9331 | | | | | | | |
| 9339 | | | | | | | |
| 9350 | 9350 | 9347 | | | | | |
| | 9362 | | | | | | |
| 9378 | | | | | | | |
| 9451 | 9449 | | | | | | |
| | | 9490 | | | | | |
| | 9660 | | 9658 | | | | |
| | | 9691 | | | | | |
| | 9731 | | | | | | |
| | | 9753 | | | | 9753 | |
| | | 9823 | 9826 | 9825 | 9826 | 9827 | |
| | | | | 9876 | | | |
| | | | | | | 9882 | |
| | 9969 | 9967 | 9969 | 9969 | 9969 | 9969 | |
| 10043 | 10045 | 10045 | | | | 10042 | 10042 |
| | | | | | | | 10067 |
| | 10101 | 10100 | 10102 | 10101 | | 10102 | 10101 |
| | | | | 10131 | | | |
| | 10241 | 10238 | | | | | 10240 |
| | | 10275 | | | | | |
| | 10371 | 10369 | | | 10370 | | |
| | | | | | | | 10412 |
| | 10453 | 10453 | | | | | |
| 10699 | 10697 | 10696 | 10697 | 10696 | 10695 | 10697 | 10696 |
| | | | | | | 10709 | 10709 |
| | | 10735 | 10738 | | | | |
| | | | | | 10764 | | |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
|  |  | 10814 |  |  |  |  |  |
|  | 10828 | 10827 | 10829 |  |  |  |  |
| 11064 | 11064 | 11064 |  |  |  |  |  |
|  |  |  |  | 11158 |  | 11159 |  |
|  | 11338 | 11336 | 11338 | 11338 | 11337 | 11337 | 11337 |
|  |  |  | 11351 | 11353 | 11349 | 11355 |  |
|  |  |  |  | 11376 |  |  |  |
|  | 11469 |  |  |  |  |  |  |
|  |  |  |  |  |  | 11859 |  |
|  |  |  |  | 11889 |  | 11888 |  |
|  |  |  |  | 12049 |  | 12046 |  |
|  |  |  | 12074 | 12073 |  | 12074 |  |
|  | 12155 | 12153 | 12154 | 12154 | 12154 | 12154 |  |
|  |  |  |  |  |  | 12180 |  |
|  |  | 12285 |  |  |  |  |  |
|  |  | 12300 |  |  |  | 12306 |  |
|  | 12410 | 12407 |  |  |  |  | 12406 |
|  |  |  |  |  |  | 12443 |  |
|  |  |  |  | 12475 |  |  |  |
|  | 12577 | 12575 | 12576 | 12578 | 12573 | 12576 | 12569 |
|  |  | 12603 |  |  | 12603 |  |  |
|  |  |  |  |  |  | 12858 |  |
|  |  |  |  |  | 13203 | 13204 | 13204 |
|  | 13226 | 13223 |  |  |  |  |  |
|  |  |  |  |  | 13336 |  |  |
|  |  |  |  |  | 13363 |  |  |
|  |  | 13535 |  |  |  |  |  |
|  |  |  |  |  |  |  | 14215 |
|  | 15240 |  |  |  |  |  |  |
|  |  |  |  | 15487 |  |  | 15486 |
|  |  |  | 15633 |  |  |  |  |
|  |  |  | 15878 |  |  |  |  |
|  |  |  | 16829 | 16826 |  |  |  |
|  |  |  | 17066 | 17066 | 17063 | 17064 |  |
|  |  |  |  | 17131 | 17125 | 17130 |  |
|  |  |  |  |  | 17154 | 17158 |  |
|  | 17353 |  | 17352 | 17349 |  |  |  |
|  |  | 17585 |  |  |  |  |  |

| 4 h | 8 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | 17791 | | | 17795 | 17789 | 17790 |
| | | | | | 18135 | | |
| | | | 19700 | | 19702 | | |
| | | | | | | | 20756 |
| | | | | | | | 20774 |
| | | | | | 22403 | 22399 | 22405 |
| | | | | | 22430 | | |

**Table A.7** $[M+H]^+$ (Da) from *Staphylococcus arlettae* (ATCC 43957) extracts by LC/off-line MALDI MS.

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| 2005 | 2006 | 2006 | 2005 | 2005 | | | |
| | 2010 | 2011 | | | 2010 | 2011 | |
| | | 2030 | 2031 | 2030 | | | |
| | | | | | | 2059 | |
| | | | | 2064 | 2065 | | |
| | | | 2082 | | | | |
| 2105 | 2105 | | 2105 | | | | |
| | | 2116 | | | | | |
| | | | 2130 | 2130 | | | |
| | | | 2141 | | | | |
| | | | 2188 | 2189 | 2188 | | |
| | | 2208 | | | | | |
| | | | 2230 | | | | |
| | | 2282 | 2282 | 2282 | | | |
| | | | | | 2315 | | |
| | | 2323 | 2323 | | | | |
| | | | 2329 | | | | |
| | | | 2335 | | | | |
| | 2355 | | | | | | |
| | | 2373 | 2372 | 2371 | 2373 | | |
| | | 2395 | 2395 | 2395 | 2396 | | |
| | | 2400 | 2400 | 2400 | 2400 | | |
| | | 2404 | 2404 | | 2405 | | |
| 2409 | | | | | | | |
| | | | | | 2416 | | |
| | | 2421 | 2420 | 2421 | | | |
| | | | 2428 | | | | |
| | | | 2432 | | | | |
| | | | 2449 | | | | |
| | 2454 | | 2453 | | | | |
| 2464 | | | | | 2466 | | 2466 |
| | 2470 | | | | | | |
| | | | 2479 | | | | |
| | | | 2483 | 2484 | | | |
| | | 2489 | | | 2488 | | 2488 |
| | | | 2522 | | 2520 | | |
| | 2545 | | 2545 | | | | |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
|  | 2568 |  |  |  |  |  |  |
|  |  |  |  |  |  | 2587 |  |
|  |  | 2592 | 2592 | 2591 | 2592 |  |  |
|  |  |  | 2604 |  |  |  |  |
|  |  |  | 2614 | 2613 |  |  |  |
|  |  |  | 2646 | 2647 |  |  |  |
|  |  |  | 2649 |  |  |  |  |
| 2669 | 2669 |  |  |  |  |  |  |
|  |  |  | 2672 |  |  |  |  |
|  |  |  | 2692 | 2692 |  |  |  |
|  |  | 2696 | 2694 |  | 2694 |  |  |
|  | 2712 |  |  |  |  |  |  |
|  |  |  | 2734 | 2734 | 2735 |  |  |
|  |  | 2738 |  |  |  |  |  |
|  |  |  | 2749 |  |  |  |  |
| 2798 | 2798 |  | 2798 | 2798 | 2798 |  |  |
|  | 2815 | 2816 |  |  |  |  |  |
|  |  | 2837 | 2836 |  |  |  |  |
|  |  | 2863 | 2863 | 2861 |  |  |  |
| 2898 | 2897 |  |  |  |  |  |  |
|  |  |  |  |  |  | 2909 |  |
|  |  | 2916 |  |  |  |  |  |
|  | 2919 |  |  | 2918 |  |  |  |
|  |  | 2924 | 2923 |  |  |  |  |
|  |  | 2944 | 2943 | 2943 | 2944 |  |  |
|  |  |  | 2963 |  | 2963 | 2964 |  |
|  |  |  |  |  |  | 2966 |  |
|  |  |  | 2969 |  |  |  |  |
|  |  |  |  |  | 2993 |  |  |
|  |  |  |  |  |  | 3025 |  |
|  |  | 3044 | 3044 |  |  | 3043 |  |
|  |  |  | 3088 | 3087 |  |  |  |
|  |  | 3114 | 3114 | 3113 | 3114 |  |  |
|  |  |  | 3126 |  |  |  |  |
|  |  |  | 3132 | 3132 |  |  |  |
|  | 3134 |  |  |  |  |  |  |
|  |  | 3138 | 3137 | 3136 | 3138 |  |  |
|  |  | 3155 |  |  |  |  |  |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | 3173 | 3172 | 3172 | 3172 | | |
| | 3205 | | | | | | |
| | | 3221 | 3221 | 3221 | | | |
| | | | | | | 3257 | |
| | | 3267 | 3268 | 3267 | | | |
| | 3317 | | | | | | |
| | | 3323 | 3322 | | | | |
| | | 3341 | | | | | |
| | | | 3353 | | | | |
| | | | 3362 | | 3363 | | |
| | | | 3368 | 3368 | | | |
| | | 3400 | 3398 | 3397 | 3400 | 3399 | |
| | | | 3403 | | | | |
| | 3414 | 3412 | 3412 | 3412 | 3412 | | |
| | | | | | 3417 | | |
| | | | | | 3434 | | |
| | | | | | | 3454 | |
| | | | 3459 | 3458 | 3459 | | |
| | | | | 3480 | | | |
| | | 3487 | | | 3486 | | |
| | | | 3499 | | 3501 | | |
| | | 3617 | | 3516 | | 3518 | |
| | 3521 | | | | | | |
| | 3533 | | | | | | |
| | | 3543 | | | | | |
| | | | 3545 | 3545 | | | |
| | 3576 | | | 3574 | 3577 | | |
| | 3606 | | | | | | |
| | 3619 | | 3618 | 3619 | | | |
| | 3634 | | | | | | |
| | | 3637 | | | 3637 | | |
| | 3649 | | | | | 3648 | |
| | 3663 | | | | | | |
| | | | 3668 | | | | |
| | 3672 | | | | | | |
| | 3676 | | 3675 | 3676 | | | |
| | 3684 | | | | | | |
| | 3720 | 3719 | 3719 | 3718 | 3719 | 3721 | |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | | 3724 | 3724 | | | |
| | | | | | | 3729 | |
| | 3740 | | | | | | |
| | 3743 | | | | | | |
| 3751 | | | | | | | |
| | | 3789 | | | | | |
| | | | | | | 3809 | |
| | 3812 | | | | | | |
| | | 3821 | 3821 | 3820 | 3821 | | |
| | | 3860 | 3859 | | | | |
| | | | | | | 3870 | |
| | 3878 | | | 3880 | 3879 | | |
| | | | | | 3911 | | |
| | | | | | | 3913 | |
| | | 3919 | 3918 | 3918 | | | |
| | | | | | | 3924 | |
| | | | | | 3934 | | |
| | | | | | | 3938 | |
| | | 3941 | 3940 | 3940 | | | |
| | | | | 3974 | | | |
| | | | 3995 | | | | |
| | 4027 | | | | | | |
| | 4089 | | 4089 | 4087 | 4086 | | |
| | | 4095 | 4096 | 4094 | 4094 | | |
| | | | | 4098 | | | |
| | | 4103 | 4104 | 4104 | 4105 | | |
| | 4112 | | | | | | |
| | 4128 | | | | | | |
| | 4140 | | 4141 | 4142 | 4144 | 4140 | |
| | 4182 | | | 4182 | | | |
| | 4198 | | | 4198 | | | |
| | | 4210 | | 4209 | 4210 | | |
| | | 4218 | 4218 | | 4219 | 4219 | 4221 |
| | | 4235 | 4235 | 4235 | | | |
| | | | | | | | 4243 |
| | | | | 4255 | | | |
| | | | | | | | 4260 |
| | | | 4269 | 4269 | | | |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | | | | | 4287 | |
| | | | 4294 | 4293 | | | |
| | 4312 | | | | | | |
| | | | 4333 | | | | |
| | | 4347 | 4348 | 4347 | 4348 | | |
| | | 4379 | 4379 | 4378 | 4378 | | |
| | | | 4387 | | | | |
| | | | | | | 4409 | |
| | 4460 | | | | | | |
| | 4465 | | | | | | |
| | | | | | | 4476 | |
| | | 4479 | | | | | |
| | | | | 4519 | | | |
| | | | | | | | 4530 |
| | 4551 | | | | | | 4553 |
| | | 4566 | | | | | |
| | | | | | | | 4570 |
| | | | | | 4576 | | |
| | | | | | 4604 | | |
| | | | | | | 4613 | |
| | | | 4616 | 4617 | | | |
| | 4620 | | 4620 | | 4620 | | |
| | | 4631 | | | | | |
| | | | 4660 | 4660 | 4661 | | |
| | | | 4684 | 4685 | | | |
| | | 4694 | 4695 | 4694 | 4695 | | |
| | | | | 4717 | | | |
| | | 4729 | 4729 | 4728 | 4728 | | |
| | | 4745 | | | | | |
| | 4775 | | | | | | |
| | | | | 4800 | 4800 | | |
| | | | 4813 | | | | |
| | | | | | 4821 | | |
| | | 4828 | 4828 | | | | |
| | 4851 | 4849 | 4852 | 4852 | | | |
| | | 4865 | 4865 | 4864 | 4865 | | |
| | | | | | 4877 | | |
| | 4885 | 4884 | | | | | |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
|  | 4900 |  |  |  |  |  |  |
|  | 4909 | 4908 | 4907 | 4907 |  |  |  |
|  |  | 4921 |  | 4921 |  |  |  |
|  |  |  |  | 4931 | 4928 | 4932 |  |
|  |  |  | 4965 | 4965 | 4966 |  |  |
|  |  |  |  |  | 4981 |  |  |
|  |  |  |  |  |  | 4994 |  |
|  | 5001 |  |  |  |  | 5003 |  |
| 5019 | 5019 | 5019 | 5019 | 5019 | 5020 | 5019 | 5019 |
|  | 5032 | 5032 | 5031 | 5030 | 5032 |  |  |
|  | 5042 |  |  |  | 5040 |  |  |
|  |  |  | 5053 | 5053 | 5054 |  |  |
|  |  |  | 5067 | 5066 | 5066 | 5067 |  |
|  |  |  |  |  | 5079 |  |  |
|  |  |  |  |  | 5109 |  |  |
|  |  |  |  |  | 5115 |  |  |
|  |  |  | 5145 |  |  |  | 5148 |
|  |  | 5162 |  | 5162 | 5163 |  |  |
|  |  |  |  |  | 5177 |  |  |
|  |  |  | 5197 | 5198 | 5198 |  |  |
|  |  |  |  |  | 5231 |  |  |
|  |  |  | 5247 |  |  |  |  |
|  | 5258 |  |  |  |  |  |  |
|  | 5269 | 5267 | 5266 | 5265 |  |  |  |
|  |  |  | 5331 |  |  |  |  |
|  |  |  | 5367 | 5364 |  |  |  |
|  |  | 5416 | 5416 | 5415 |  |  |  |
|  |  | 5454 | 5454 |  |  |  |  |
|  |  | 5478 | 5478 |  |  |  |  |
|  |  |  |  |  | 5562 |  |  |
|  | 5607 |  |  |  |  |  |  |
|  |  |  | 5632 | 5631 | 5633 |  |  |
|  | 5650 |  |  |  |  |  |  |
|  |  |  | 5672 | 5672 |  |  |  |
|  |  |  |  | 5676 |  |  |  |
|  |  |  |  |  | 5740 |  |  |
|  | 5784 | 5785 | 5784 | 5784 | 5785 | 5785 |  |
|  |  |  |  |  |  |  | 5794 |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | | 5798 | | | | |
| | | 5826 | 5825 | 5825 | | | |
| | | | 5842 | | | | |
| | 5857 | | 5856 | 5857 | 5856 | | |
| | | | 5884 | 5883 | | | |
| | | | 5942 | | | | |
| | | | 5984 | | | | |
| | | 6080 | 6082 | 6083 | | | 6081 |
| | | | 6098 | | | | |
| | | | | | 6141 | | |
| | | | | | 6266 | | |
| | | | | | | 6299 | |
| | | | 6340 | | | | |
| 6369 | | | | | 6371 | | |
| 6383 | 6383 | 6382 | 6382 | 6382 | | | |
| | 6430 | | | | 6429 | | |
| | | | | | 6434 | | |
| | | | 6485 | 6484 | 6487 | | |
| | | 6550 | 6550 | | | 6549 | |
| | | | | | | | 6584 |
| | | 6612 | 6612 | 6612 | 6613 | | |
| | | 6641 | | 6641 | | 6641 | 6640 |
| | | 6648 | | | 6648 | | |
| | | | | | | | 6652 |
| | | | | | | 6656 | |
| | | | | 6670 | | | |
| | | 6691 | 6692 | 6692 | 6693 | 6693 | |
| | | 6705 | | | | 6708 | |
| | | | | | | | 6732 |
| | | | | | | 6741 | 6742 |
| | | | 6748 | | 6748 | | |
| | | | | | | 6757 | |
| | | | | | | | 6771 |
| | | 6804 | 6805 | 6806 | | | |
| | | | | | | | 6818 |
| | | | 6865 | | | | |
| 6901 | | 6898 | | 6898 | 6899 | 6900 | |
| | 6909 | 6908 | 6909 | 6908 | 6909 | | |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
|  |  |  | 6923 |  |  |  |  |
|  |  |  |  |  | 6945 |  |  |
|  |  |  | 6952 |  |  |  |  |
|  | 7006 |  |  |  |  | 7005 |  |
|  |  |  |  | 7077 |  |  |  |
|  |  | 7145 | 7145 | 7145 | 7145 |  |  |
|  |  | 7181 |  |  | 7180 |  |  |
|  |  |  |  |  |  |  | 7209 |
|  |  |  |  |  |  |  | 7216 |
|  |  |  |  |  |  |  | 7235 |
|  |  |  |  |  |  |  | 7294 |
|  |  | 7311 | 7311 | 7310 | 7312 |  | 7307 |
|  |  |  | 7325 |  |  |  |  |
|  |  |  |  | 7334 |  |  |  |
| 7380 |  |  |  | 7380 |  |  |  |
|  |  |  |  | 7391 | 7389 |  |  |
|  |  |  |  | 7430 | 7430 |  |  |
|  |  |  | 7576 | 7575 |  |  |  |
|  |  | 7602 | 7602 | 7602 |  |  |  |
|  |  |  |  | 7630 | 7630 |  |  |
|  |  |  |  |  | 7635 |  |  |
|  |  |  |  | 7649 | 7649 | 7649 |  |
|  |  |  |  |  |  |  | 7657 |
|  |  |  |  | 7693 |  |  |  |
|  |  |  |  |  |  |  | 7699 |
|  |  |  |  | 7710 |  |  |  |
|  |  | 7749 | 7749 | 7749 | 7750 |  | 7754 |
|  |  |  |  |  |  |  | 7803 |
| 8003 |  |  |  |  |  |  | 8002 |
|  |  | 8035 | 8033 | 8033 |  | 8035 |  |
|  |  |  |  | 8051 | 8052 |  |  |
|  |  |  |  |  |  |  | 8095 |
|  |  |  |  |  |  |  | 8104 |
|  |  |  |  | 8112 |  |  |  |
|  |  |  | 8123 | 8122 |  |  |  |
|  |  |  |  |  |  | 8151 |  |
|  |  |  |  |  |  |  | 8272 |
|  |  |  |  | 8281 |  |  |  |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | | | | | | 8300 |
| | | | | | | | 8384 |
| | | | | | | | 8455 |
| | | | | | | | 8484 |
| | | | | | | | 8588 |
| | | | | | | 8604 | |
| | | | | | | | 8643 |
| | | | | 8686 | | | |
| | | | | | | | 8789 |
| | 8946 | 8947 | 8948 | 8947 | 8947 | | |
| | | | | | | | 9009 |
| | | | 9177 | | | | |
| | | | | 9259 | 9260 | | |
| | | | | | | | 9313 |
| | | | | | | | 9319 |
| | | | | | 9516 | | |
| 9642 | | 9642 | 9642 | 9642 | 9642 | 9642 | 9641 |
| | | 9657 | 9656 | | | | 9658 |
| 9671 | | 9669 | 9670 | 9670 | 9671 | 9669 | 9670 |
| | | | | | | | 9703 |
| | | 9715 | | | | | |
| 9743 | | | | 9740 | | | |
| | | | | | 9802 | | |
| | | | | 9823 | | | 9820 |
| | | | | | | | 9830 |
| 9853 | | | | 9852 | | | 9855 |
| | | | | 9949 | | | |
| | | | | | 10007 | | 10005 |
| | | | | | 10232 | | |
| | | | | | | | 10283 |
| | | | | 10333 | | | |
| | | | | | | | 10427 |
| | | | | 10447 | | | |
| | | | | | 10519 | | |
| | | | | | | | 10540 |
| | | | | 10860 | | 10859 | |
| | | | | | 10996 | | |
| | | | | | | | 11181 |

| 4 h | 5 h | 12 h | 24 h | 48 h | 72 h | 144 h | 288 h |
|---|---|---|---|---|---|---|---|
| | | | | | | | 11226 |
| | | | | | | | 11286 |
| | | | | | | | 11295 |
| | | | | | | | 11308 |
| | | | | | | | 11354 |
| | 11369 | | | | | | |
| 11651 | | | 11651 | 11649 | 11651 | | |
| | | | | | | | 11757 |
| | | | | | | | 12161 |
| | | | | | | | 12176 |
| | | | | | | | 12188 |
| | | | | | 12360 | | |
| | | | | | | | 12422 |
| | | | | | | | 13373 |
| | | | | 15196 | | | |
| | | | | | 15433 | | |
| | | | | | | | 15851 |
| | | | | | | | 16633 |
| | | | | | | | 20682 |

**Table A.8** [M+H]$^+$ (Da) from *Staphylococcus kloosii* (ATCC 43959) extracts by LC/off-line MALDI MS.

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | | 2006 | 2005 | 2006 | | | | | | |
| 2010 | | 2011 | | 2011 | 2011 | 2010 | | | | |
| 2017 | | | | | | | | | | |
| 2030 | | | | | | | | | | |
| | | 2081 | | | | | | | | |
| 2085 | 2084 | 2085 | 2084 | | | | | | | |
| | 2105 | | | | 2105 | 2105 | | | | |
| | | | | 2111 | 2110 | 2110 | 2110 | | | |
| | | | 2115 | | | | | | | |
| 2155 | | | | | | | | | | |
| 2160 | | | | | | | | | | |
| 2173 | | | | 2173 | 2173 | | | | | |
| | | 2176 | | | | | | | | |
| 2184 | 2185 | 2187 | 2183 | 2186 | | 2185 | | | | |
| 2196 | | | | 2197 | 2197 | | | | | |
| | | | | | | 2206 | | | | |
| | | | | | | | | 2216 | | 2214 |
| | | | | 2226 | | | | | | |
| 2283 | | | | | | | | | | |
| | | | | | 2324 | | | | | |
| 2335 | | | | | | | | | | |
| 2355 | | | 2354 | 2354 | | | | | | |
| | | | | 2392 | 2391 | | | | | |
| 2397 | | | | | | | | | | |
| 2411 | | | | | | | | | | |
| | 2455 | | | 2454 | | | | | | |
| 2433 | | | | | | | | | | |
| | | | 2453 | | 2454 | | | | | |
| 2458 | | | | | | | | | | |
| | | 2466 | | | | | | 2465 | | |
| | | | 2469 | | | | | | | |
| | | | | 2494 | | | | | | |
| 2545 | 2545 | | | 2546 | 2546 | | | | | |
| | | | | 2601 | | | | | | |
| 2610 | 2611 | | | | | 2611 | | | | |
| 2645 | | | | | | | | | | |
| 2649 | | | | | | | | | | |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  | 2669 |  |  | 2669 |  |  |  |  |
|  | 2673 |  |  |  |  |  |  |  |  |  |
| 2712 |  |  |  |  | 2712 |  |  |  |  |  |
| 2737 |  |  |  |  |  |  |  |  |  |  |
| 2745 |  |  |  |  |  |  |  |  |  |  |
| 2763 |  |  |  |  |  |  |  |  |  |  |
| 2768 |  |  |  |  |  |  |  |  |  |  |
| 2774 |  |  |  |  |  |  |  |  |  |  |
| 2782 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 2787 |  |  |  |  |  |
| 2799 | 2799 | 2798 | 2799 | 2799 | 2799 | 2799 |  |  |  |  |
|  | 2815 | 2816 |  |  |  |  |  |  |  |  |
| 2827 |  |  |  |  |  |  |  |  |  |  |
| 2837 |  |  |  |  |  |  |  |  |  |  |
| 2845 | 2845 |  |  |  |  |  |  |  |  |  |
| 2854 |  |  |  |  |  |  |  |  |  |  |
| 2857 |  |  |  |  |  |  |  |  |  |  |
| 2863 |  |  |  |  |  |  |  |  |  |  |
| 2879 |  |  |  |  |  |  |  |  |  |  |
| 2898 |  |  |  |  |  |  |  |  |  |  |
|  | 2915 | 2915 | 2915 | 2916 |  |  |  |  |  |  |
|  |  |  |  |  |  | 3060 |  |  |  |  |
| 3066 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  | 3083 | 3082 | 3083 |  |  |  |  |
|  |  |  |  |  | 3128 | 3127 | 3127 |  |  |  |
|  |  |  | 3171 |  |  |  |  |  |  |  |
|  |  |  |  | 3184 |  | 3184 |  |  |  |  |
|  | 3198 |  |  |  |  |  |  |  |  |  |
|  |  |  |  | 3220 | 3220 |  |  |  |  |  |
|  |  |  |  |  | 3237 |  |  |  |  |  |
| 3241 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  | 3248 | 3247 |  |  |  |  | 3426 |
|  |  | 3309 |  |  |  |  |  |  |  |  |
| 3335 |  |  |  | 3333 |  |  |  |  |  |  |
| 3340 | 3342 | 3342 |  |  |  |  |  |  |  |  |
| 3390 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 3403 |  |  |  |  |  |
| 3454 |  |  |  |  |  |  |  |  |  |  |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| 3547 | | | | | | | | | | |
| | | | | | 3676 | | | | | |
| 3733 | | | | | | | | | | |
| | | | | | 3747 | 3746 | | | | |
| 3762 | | | | | | | | 3763 | | |
| 3784 | | | | | | | | | | |
| | | | | | | | | | 3880 | 3882 |
| | | | | | | | | | | 4088 |
| | | | | 4175 | 4174 | 4173 | | | | |
| | | | | | | | | 4190 | | |
| 4332 | | | | | | | | | | |
| | | | 4373 | | 4375 | 4375 | 4374 | | | |
| | | | | | 4456 | | | 4457 | | |
| | | | | | | | | | | 4512 |
| | | | | | 4595 | 4595 | | | | |
| | | | | | | 4623 | | | | |
| | | | | | | | | | | 4626 |
| | | | | | | | | | 4615 | |
| | | | | 4670 | 4670 | | | | | |
| | | | | | | | | | | 4775 |
| | | | | | 4862 | | | 4865 | | |
| | | | | | | | | | 4870 | |
| | | | | | | 4902 | 4902 | | 4902 | 4902 |
| | | | | | | | 4938 | | | |
| | | | 4943 | 4944 | 4945 | 4944 | | 4945 | | |
| | | | | | | | 4958 | | | |
| | | | | | | | 4970 | 4967 | | |
| | | 4989 | 4989 | 4989 | 4989 | 4989 | 4989 | 4989 | 4990 | 4991 |
| | | | | 5011 | | | | | | |
| | | | 5029 | | | | | | | |
| | | | 5045 | 5048 | 5048 | 5046 | 5047 | | | |
| | | | | | | 5067 | | | | |
| | | | | | 5072 | | | | | |
| | | | | | 5085 | 5086 | | | | |
| | | | | | 5090 | | | | | |
| | | | | 5095 | 5094 | 5095 | | 5095 | 5095 | 5098 |
| | | | | | | | | | 5105 | |
| | | | 5120 | | 5121 | 5119 | | | | |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5133 | | | | | | | |
| | | | | | | 5137 | | | | |
| | | | | | | 5158 | | | | |
| | | | | 5171 | 5173 | | | | | |
| | | | 5225 | 5226 | 5226 | 5225 | | | | |
| | | | 5240 | | | | | | | |
| | | | | | | | | | 5336 | |
| | | | 5381 | | | | | | | |
| 5433 | | | | 5435 | 5434 | | | | 5433 | |
| | | | | | | | | | | 5499 |
| | | | | | | | 5585 | | | |
| | | | 5591 | | | | | | | |
| | | | 5602 | 5603 | 5602 | 5601 | 5602 | 5603 | 5604 | |
| | | | | | | | | | 5626 | |
| 5693 | | | | | | | | | | |
| 5716 | | | | | | | | | | |
| 5727 | | | | | | | | | | |
| | | | 5794 | | | | | | | |
| | | | 5805 | | | | | | | |
| | | | | | 5993 | 5993 | | | | 5994 |
| | | | | | | | | | | 6010 |
| | | 6110 | | | | | | | | |
| | | | | | | | | 6114 | 6115 | |
| | | | | | 6117 | | | 6118 | 6118 | 6119 |
| | | | | | | | | | 6214 | |
| | | | | | | | 6345 | 6344 | 6344 | |
| | | 6353 | | | 6353 | | | | | |
| | 6369 | | 6368 | 6370 | 6369 | 6369 | | | | |
| 6396 | 6397 | 6397 | 6397 | 6397 | 6396 | 6396 | 6396 | 6397 | 6396 | 6397 |
| 6410 | | | 6410 | | | | | | | |
| | | | 6435 | | | | | | | |
| | | | | | | 6439 | 6440 | | 6438 | 6441 |
| 6455 | | 6454 | | | 6455 | | | | | |
| | | | | | | | | | | 6468 |
| | | | | | 6488 | 6489 | 6488 | 6488 | 6487 | 6488 |
| | | 6497 | | | | | | | | |
| | | 6511 | 6511 | 6512 | 6511 | 6510 | 6511 | 6510 | 6509 | 6512 |
| | | 6544 | | | | | | | | |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 6554 | |
| | | | | | | | | 6561 | | |
| | | | | | 6566 | 6566 | | | | |
| | | | | 6573 | | | | | 6570 | |
| | | | | 6585 | 6584 | 6585 | 6586 | 6585 | 6587 | 6590 |
| | | | | | | 6605 | | | | |
| | | | | | | | 6619 | 6619 | | 6619 |
| | | 6642 | 6642 | 6643 | 6642 | 6641 | | | | |
| | | | | | | 6654 | | | | |
| | | | | | | 6659 | | | | |
| | | | | | 6693 | 6692 | | | | |
| | | | | | | 6720 | 6720 | | | |
| | | | | | | | 6738 | 6739 | 6738 | 6739 |
| | | | 6804 | | | | | | | |
| | | | | | | | 6831 | | | |
| 6869 | | 6870 | 6868 | | 6869 | 6871 | 6869 | 6869 | 6869 | 6869 |
| 6902 | | | | | | | | | | |
| | | 6944 | 6943 | 6944 | 6943 | 6944 | 6944 | | | 6941 |
| 6952 | | | 6951 | | | | | | | 6951 |
| | | | | | | | 6972 | 6973 | | |
| | | | | | 6995 | 6993 | | | | |
| | | | 7004 | | | 7000 | | | | |
| 7035 | | 7036 | 7036 | 7036 | 7036 | 7037 | 7036 | 7036 | 7036 | |
| | | | | | | | | 7045 | | |
| | | | 7051 | | | 7052 | | | | |
| | | | | | | 7060 | | | | |
| | | | | | | 7088 | | | | |
| | | | | | | 7093 | | | | |
| | | 7167 | 7167 | 7168 | 7167 | 7168 | | | | |
| | | | | | | | 7201 | | | |
| | | | | | | | | 7224 | 7225 | 7225 |
| | | | | | | | | | 7240 | |
| 7351 | 7351 | 7351 | 7351 | 7351 | 7350 | 7351 | 7352 | 7352 | 7352 | 7354 |
| | | | 7366 | | | | | | | |
| 7379 | 7380 | 7382 | | | 7379 | | 7380 | 7380 | 7380 | 7381 |
| | 7428 | | | | | | | | | |
| | | | | | | | 7437 | | | |
| | | | | | | | | 7446 | | |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  | 7450 |  |  |  | 7452 | 7451 |  |  |
|  |  |  | 7467 |  |  |  |  |  |  |  |
|  |  | 7482 | 7482 | 7483 | 7482 |  |  |  |  |  |
|  |  |  | 7496 |  |  |  |  |  |  |  |
| 7512 |  | 7511 | 7511 |  | 7512 | 7511 |  | 7512 |  | 7513 |
|  |  |  |  |  |  |  | 7523 | 7521 |  |  |
|  |  |  | 7542 |  |  |  |  |  |  |  |
|  |  |  | 7548 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 7601 |
|  |  |  | 7609 |  |  |  |  |  |  |  |
|  |  | 7617 |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 7632 | 7630 |  | 7633 |
|  |  |  |  |  |  |  |  |  |  | 7646 |
|  |  |  |  |  |  |  |  | 7856 | 7856 |  |
|  |  |  |  |  |  |  |  | 7880 | 7879 |  |
|  |  |  |  |  |  |  | 7968 | 7970 | 7969 |  |
|  |  |  |  |  |  |  | 8018 |  |  |  |
|  |  |  |  |  | 8032 |  |  |  |  |  |
|  |  |  |  |  |  |  | 8049 |  |  |  |
| 8060 | 8061 |  |  |  |  |  | 8061 | 8061 | 8060 | 8062 |
| 8089 | 8091 |  |  |  |  |  | 8089 |  |  |  |
|  |  |  |  |  |  |  | 8096 |  |  |  |
|  |  |  |  |  |  |  | 8102 | 8105 |  |  |
|  |  |  |  |  |  |  |  |  |  | 8104 |
| 8149 | 8148 |  |  |  |  |  | 8149 | 8150 |  |  |
|  |  |  |  |  |  |  | 8159 |  |  |  |
| 8270 |  |  |  |  |  |  |  |  |  |  |
| 8316 |  |  |  |  |  |  |  |  |  |  |
| 8346 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 8396 |  |  |
|  |  |  |  |  |  |  | 8541 | 8540 | 8540 | 8540 |
|  |  |  |  |  |  |  | 8599 |  |  |  |
|  |  |  |  |  |  |  |  |  | 8734 |  |
| 8819 | 8814 |  |  |  |  |  |  | 8820 |  |  |
| 8850 |  |  |  |  |  |  |  | 8847 |  |  |
| 8859 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  | 8865 | 8863 |
| 8966 |  |  |  |  |  |  |  | 8966 | 8967 |  |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 8976 | 8976 | 8977 | 8977 | 8977 | 8979 | |
| 9010 | | | | | | | | | | |
| | 9027 | 9025 | 9026 | 9026 | 9026 | 9026 | | | | 9026 |
| | 9039 | | | | | | | | | |
| | | | | | | | | | | 9542 |
| 9583 | | | | | | | 9584 | 9583 | 9583 | |
| 9673 | 9671 | | 9670 | | 9672 | 9672 | 9671 | 9671 | 9671 | 9672 |
| | | | 9698 | | | | | | | |
| | | | 9713 | | | | | | | |
| | | | | | 9741 | 9738 | 9741 | 9742 | 9741 | 9743 |
| | | | | | | 9758 | | | | |
| 9764 | | 9764 | 9763 | 9765 | 9764 | 9763 | 9764 | 9764 | 9764 | 9767 |
| | 9877 | | | | | | 9879 | | | |
| 9888 | | | | | | | | 9888 | 9891 | 9890 |
| | | 9895 | 9895 | | 9895 | | 9894 | | | |
| 9941 | | | | | | | 9942 | 9942 | 9941 | 9941 |
| | | | | | | | | 9959 | | |
| | | | | | | | 9989 | | | |
| 10022 | | | 10021 | | 10019 | 10020 | 10021 | 10021 | 10020 | 10022 |
| | | | | | | | | | 10036 | |
| | | | | | | | | | 10066 | |
| | | | | | | | 10089 | | | |
| | | 10114 | | | | | | | | |
| 10153 | | | 10152 | 10151 | 10151 | 10152 | 10152 | 10153 | 10152 | |
| | | | | | | | | 10162 | | 10163 |
| | | | | | | | 10180 | | | |
| 10234 | | | | | | | | | | |
| | | | | | | | 10272 | 10272 | 10272 | 10274 |
| | | | | | | | 10361 | 10366 | | 10367 |
| | 10425 | | | | | | 10429 | | | |
| | | | | | | | | 10588 | | |
| | | | | | | | | | | 10711 |
| | | | | | | | 10793 | | 10787 | 10789 |
| 10841 | | | | | | | 10843 | 10845 | 10842 | 10843 |
| | | | | | | | 10871 | | | |
| | | | | | | | 10918 | | 10920 | |
| | | | | | | | 10926 | | | |
| | | | | | | | | 11024 | | |

| 3 h | 4 h | 6 h | 12 h | 24 h | 48 h | 72 h | 144 h | 192 h | 292 h | 484 h |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 11066 | 11065 | 11066 | |
| 11148 | | | | | | | 11150 | 11149 | 11150 | 11153 |
| 11277 | | | | | | | | 11279 | 11277 | |
| | | 11589 | 11589 | | 11590 | | 11590 | | | 11585 |
| 11912 | | | | | | | | 11912 | 11912 | |
| 12054 | | | | | | | | | | |
| 12129 | | | | | | | | 12128 | | |
| 12323 | 12323 | | | | | | 12320 | 12324 | 12322 | |
| 12787 | | | | | | | | | | |
| 13217 | 13216 | | | | | | | 13218 | 13216 | |
| | | | | | | | 13345 | 13345 | 13346 | |
| | | | | | | | | 13561 | | |
| | | | | | | | | | | 14738 |
| | | | | | | | 14894 | | | |
| | | | | | | | | 15054 | 15050 | |
| | | | | | | | | | | 15943 |
| | | | | | | | 15957 | | | |
| | | | | | | | 16327 | | | |
| | | | | | | | | 16392 | 16392 | |
| 17721 | | | | | | | | | | |
| | | | | | | | 17833 | 17826 | 17820 | |
| 18022 | | | | | | | | 18021 | 18019 | |
| | | | | | | | | 20200 | | |
| 23423 | | | | | | | | | | |
| | | | | | | | | | 24387 | |
| | | | | | | | | 24420 | 24426 | |
| 24519 | | | | | | | | | | |
| | | | | | | | 30011 | 30005 | 30014 | |
| | | | | | | | 30029 | 30025 | 30026 | |

**Table A.9** [M+H]$^+$ (Da) from *Streptococcus bovis* (ATCC 33317) extracts by LC/off-line MALDI MS.

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | 2052 | | |
| | 2150 | | | | | 2151 | |
| | 2167 | | | | 2167 | 2168 | |
| | | 2184 | 2184 | | | | |
| | | | | | | | 2228 |
| | | | | | 2280 | 2281 | |
| 2381 | | 2382 | 2382 | | 2380 | | |
| | | | | | | 2395 | 2395 |
| 2411 | | 2412 | | | 2411 | 2412 | 2412 |
| | 2452 | 2452 | | | 2451 | 2452 | |
| 2469 | 2469 | 2469 | 2469 | | 2468 | 2469 | |
| | | 2511 | 2512 | | | | |
| | | | | | 2525 | | 2524 |
| | | | | | | 2570 | 2570 |
| | | | | | | 2577 | |
| 2583 | 2583 | 2582 | 2582 | | 2582 | | |
| | | | | | | | 2599 |
| | | 2611 | 2610 | | | | |
| | 2637 | | 2637 | | | | |
| 2654 | | | | | | | |
| 2675 | | | | | | | 2674 |
| | | | 2745 | | | | |
| 2794 | 2794 | | 2794 | | | | |
| | | | 2802 | | | | |
| | | 2853 | | | | | |
| 2881 | | | | | | | |
| | | 2927 | | | | | |
| | | | | | | 2934 | |
| | | | | | | | 3222 |
| 3279 | | | | 3279 | | | |
| 3283 | | | | | | | |
| | | | | | | | 3302 |
| | | 3316 | | | | | |
| | | 3351 | 3350 | | | | |
| | | | | 3372 | | | |
| | 3440 | | | | 3440 | | |
| | | | | 3443 | | | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | 3455 | 3453 | | | | |
| 3460 | | | | | | | |
| 3469 | | | | | | | |
| | | | | 3487 | | | |
| 3519 | 3519 | 3519 | 3518 | | | 3519 | 3518 |
| | | | | | | | 3524 |
| | 3530 | 3529 | 3530 | | | 3531 | |
| 3533 | | | | | | | |
| | | 3543 | 3543 | | | | |
| 3549 | | 3548 | 3548 | | | | |
| 3563 | 3562 | 3563 | 3562 | 3563 | 3561 | 3563 | 3562 |
| 3577 | 3577 | 3576 | 3576 | | 3576 | 3577 | |
| 3606 | | | | | | | |
| 3620 | 3620 | 3620 | 3620 | 3620 | 3619 | 3621 | 3620 |
| 3649 | | 3649 | 3650 | | | | |
| 3663 | 3664 | 3663 | 3663 | | | | |
| 3677 | 3678 | 3678 | 3677 | 3678 | 3676 | 3678 | |
| 3696 | | | | | | | |
| | | | 3764 | | | | |
| | | | | | | 3776 | |
| | | 3778 | | | | | |
| | 3809 | | | | | | |
| | | 3828 | | | | | |
| | | 3839 | | | | | |
| | 3869 | | | | | | |
| 3873 | | | | | | | |
| 3903 | | | | | | | |
| | | | 3908 | | | | |
| 3954 | | | | | | | |
| | | | | | | | 4009 |
| 4015 | | 4016 | 4015 | 4015 | 4015 | | |
| | | | | | | 4036 | |
| 4086 | | 4087 | | | | | |
| | | | 4103 | | | | |
| | | | | | | | 4125 |
| | | | | 4129 | | | |
| 4143 | | | 4142 | 4143 | | | |
| 4149 | 4147 | | 4149 | | | 4149 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | | 4206 | |
| 4214 | | | | | | | |
| 4223 | | | | | | | |
| | | 4248 | 4247 | 4246 | 4246 | 4246 | 4244 |
| 4303 | | | | | | | |
| | | | 4365 | | 4365 | | |
| 4378 | 4380 | | 4378 | 4379 | 4378 | 4379 | |
| | | | | | 4393 | | |
| | | | | | | 4434 | |
| 4437 | 4439 | 4437 | 4438 | | 4437 | | |
| 4441 | | | | | | | |
| | | 4454 | | | | | |
| 4476 | | | | | | 4476 | |
| | 4490 | 4490 | | | | | |
| 4494 | | | | | | | |
| | | | | | | 4505 | |
| | | | | 4510 | | | |
| | | | | 4526 | 4527 | 4526 | |
| | | | 4570 | 4568 | | | |
| 4588 | 4585 | 4584 | | | | | |
| 4591 | 4591 | 4591 | 4591 | 4592 | 4591 | 4591 | |
| | | | | | | 4605 | |
| | | | | | | | 4609 |
| | | | | | | 4617 | |
| | | | 4626 | | | | |
| | | | | | 4648 | | |
| | | | 4671 | | | | |
| | | 4685 | | | | | |
| 4713 | 4713 | 4715 | | | 4712 | | |
| | | | | | 4750 | 4752 | |
| 4789 | 4789 | 4789 | 4788 | 4787 | 4788 | 4788 | |
| 4812 | | 4810 | 4809 | | 4809 | 4810 | |
| | | | | | | 4832 | |
| 4847 | 4844 | | 4845 | | | 4847 | |
| 4858 | | 4857 | | | | | |
| | | | 4863 | | | | |
| | 4873 | | | | | | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
|  |  |  |  |  | 4893 |  |  |
|  |  |  |  |  |  | 4904 |  |
| 4961 |  | 4961 | 4961 |  |  |  |  |
|  |  |  |  |  |  | 4979 |  |
|  | 5008 |  |  |  |  |  |  |
| 5016 |  |  |  |  |  |  |  |
|  |  | 5020 | 5019 |  |  |  |  |
| 5052 |  |  |  |  | 5052 |  |  |
|  | 5071 | 5071 | 5071 |  |  |  |  |
| 5114 |  |  |  |  |  |  |  |
|  | 5120 | 5120 |  |  |  |  |  |
|  |  |  |  | 5133 |  |  |  |
| 5157 |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 5165 |  |
| 5171 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 5189 |
| 5199 | 5200 | 5202 |  |  |  |  |  |
|  | 5221 | 5221 |  |  |  |  |  |
|  |  |  |  | 5234 |  |  |  |
| 5238 |  |  |  |  | 5238 | 5238 |  |
|  | 5255 |  |  |  |  |  |  |
|  |  |  |  |  |  | 5273 |  |
| 5298 |  |  |  |  |  |  |  |
|  |  |  |  | 5301 |  |  |  |
|  |  | 5314 |  |  |  |  |  |
|  |  |  |  |  | 5338 |  |  |
| 5363 |  |  |  | 5363 | 5363 |  |  |
|  |  |  |  | 5381 |  |  | 5379 |
|  |  |  |  |  |  | 5408 | 5407 |
| 5420 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 5423 |
| 5431 |  |  |  |  |  |  |  |
|  |  |  |  |  | 5440 |  |  |
|  |  |  |  |  | 5456 | 5455 |  |
|  | 5483 |  |  |  | 5482 |  |  |
|  |  | 5489 |  |  |  |  |  |
|  | 5555 |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 5563 |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 5574 | | | | | | | |
| | | | | | 5586 | | |
| | | | | | | | 5606 |
| | | | | | | | 5622 |
| | | | | | | 5629 | |
| | | 5696 | | | | | |
| | | | | | 5714 | | |
| | | | | | 5733 | | 5734 |
| 5737 | 5737 | 5736 | 5738 | 5737 | 5736 | 5738 | |
| | | | | 5754 | | | |
| 5759 | | | | | | | |
| 5764 | | | | | | | |
| | | | | | | 5770 | |
| 5777 | | | | | | | |
| | | | | | | 5794 | |
| | | | 5802 | | | | |
| | | | | | | 5850 | |
| | | 5894 | | | | | |
| 5926 | 5927 | | 5927 | | | | |
| 5946 | | | | | | | |
| 5954 | 5955 | 5954 | | | | | |
| | 5961 | 5962 | 5960 | 5961 | | 5964 | |
| 5971 | 5970 | 5968 | 5968 | 5968 | 5968 | | 5967 |
| | | | | | 5975 | | |
| | | | | | | 5982 | |
| 5986 | | | | | | | |
| | | | | | 6024 | | |
| | | 6042 | | | | | 6040 |
| | | 6051 | | | | | |
| | | | | | 6065 | | |
| | | | 6069 | | | | |
| | | | 6086 | | | | |
| 6091 | | | | | | | |
| 6114 | 6113 | 6113 | 6113 | 6112 | | | 6113 |
| 6130 | | | | | 6130 | 6129 | |
| 6140 | 6141 | | 6140 | | | | |
| 6170 | | | | | | | |
| | | | 6176 | | | | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | | 6206 | |
| | | | 6213 | | | | 6216 |
| | 6234 | 6233 | 6234 | 6232 | 6230 | | |
| 6242 | | 6242 | 6241 | 6241 | 6241 | | |
| 6249 | 6249 | 6250 | 6248 | 6247 | 6247 | | 6246 |
| 6253 | | | | | 6251 | | |
| 6257 | | | | | | | |
| | | | 6282 | | | | |
| | | 6338 | 6335 | | | | |
| | | | 6347 | 6344 | | | |
| 6369 | | | | | | | |
| | | | | 6378 | | | |
| | | | | | 6402 | | |
| | | | 6413 | | | | |
| | | | | | 6458 | | |
| 6492 | 6487 | | 6491 | | 6491 | 6492 | 6492 |
| 6498 | | 6497 | | | 6499 | | |
| 6521 | | | | | | | |
| 6528 | | | | | 6525 | | |
| | 6533 | | | | | | |
| | 6552 | 6553 | 6552 | 6553 | 6553 | | |
| | 6615 | | | | | | |
| 6611 | | 6611 | 6610 | | 6610 | 6610 | 6609 |
| | | | | | | | 6624 |
| | | | | | 6631 | | 6631 |
| | 6640 | | | | | | 6640 |
| | 6694 | | | | | | |
| | | | | | 6700 | | |
| | 6717 | | 6715 | | | | |
| 6725 | 6728 | | | | 6726 | 6725 | |
| | | | | | 6745 | | 6745 |
| 6754 | 6748 | 6749 | 6751 | | | | |
| | | | | | | | 6756 |
| | | | | | | 6762 | 6760 |
| | 6771 | 6771 | 6770 | | 6771 | | |
| 6776 | | | | | | | |
| | | | | | | 6790 | |
| 6794 | 6798 | 6800 | 6798 | | 6799 | 6799 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | | 6815 | 6814 |
| | | | | | 6834 | 6834 | 6832 |
| 6839 | 6839 | | 6837 | 6839 | 6842 | 6841 | 6840 |
| | | | | | | 6847 | 6847 |
| | | | | | 6862 | 6861 | |
| 6872 | 6870 | 6872 | 6871 | | | | |
| 6882 | 6882 | | | | | | |
| | 6891 | 6892 | | | 6891 | | |
| | | | | | | 6899 | 6896 |
| | | | | | 6942 | 6938 | |
| 6945 | | 6947 | 6948 | 6945 | 6946 | 6945 | 6945 |
| | | | | 6951 | | | |
| | 6961 | | | | | | |
| | | | | | | 6978 | |
| 6991 | 6991 | | 6990 | 6990 | 6990 | | |
| | | | | | | 6997 | 6978 |
| | | | | | 7006 | | |
| | | | | | | 7063 | |
| | 7076 | 7076 | 7076 | 7077 | 7075 | | |
| 7081 | 7082 | | | | | 7081 | |
| | | | | | 7088 | | 7085 |
| | | | 7092 | 7090 | 7093 | | |
| 7096 | | | | 7097 | | 7096 | |
| | | | | | | | 7105 |
| 7123 | | 7121 | | | | | |
| | | | | | 7138 | | |
| | 7146 | | | | | | |
| | | | | | | | 7154 |
| | | | | | 7164 | | |
| | | | | | | 7172 | 7170 |
| | | | 7187 | | | | |
| | | | | | 7223 | | 7223 |
| | | | | | | 7294 | |
| | | | | | 7324 | | |
| | | | | | 7351 | | |
| | 7363 | | 7363 | | | | |
| 7407 | | 7408 | | | | 7405 | 7406 |
| | | | | | | | 7420 |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | 7452 | | |
| | | | 7466 | 7466 | 7467 | | |
| | 7513 | | | | | | |
| 7546 | 7545 | | | | 7544 | 7546 | |
| | 7553 | | | | | | |
| | | | | | 7562 | | |
| 7573 | 7571 | | | | 7570 | 7571 | |
| 7596 | 7596 | | 7596 | 7597 | 7598 | 7597 | |
| | | | | | 7637 | | |
| | | | | | 7642 | | |
| | | | | | | | 7659 |
| | 7683 | | | | 7683 | | 7683 |
| | 7700 | | | | | | |
| | | | | 7707 | | | |
| 7766 | 7764 | 7765 | 7765 | | | 7765 | 7765 |
| | 7812 | | | | | | |
| | 7830 | | | | | | |
| | 7879 | | 7879 | | | | |
| | | | 7907 | 7907 | | | |
| | | | | | | | 7923 |
| | | | | | 7939 | | |
| 7945 | 7945 | 7945 | 7945 | 7944 | | 7945 | 7944 |
| | | | | | 7959 | | |
| 7972 | | | | | | | |
| 7977 | 7976 | | | | 7975 | | |
| | | | | | 7990 | 7989 | |
| | | | | 8000 | | | |
| | | | | | | | 8058 |
| | 8073 | | | | 8076 | | |
| | | | | | | | 8128 |
| | | | 8141 | 8141 | 8143 | 8142 | |
| | 8214 | | | | 8214 | | |
| 8219 | | | | | | | |
| | | | | | 8228 | | |
| | | | | | 8234 | | |
| 8250 | | | | | | | |
| 8293 | 8294 | 8293 | 8293 | 8293 | 8294 | 8293 | 8292 |
| 8304 | 8303 | 8306 | 8304 | 8303 | 8305 | 8304 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  |  | 8328 |
| 8352 | 8351 | 8352 | 8351 | 8351 | 8349 | 8351 |  |
| 8410 |  |  |  |  |  |  |  |
| 8435 | 8434 | 8437 | 8436 | 8434 |  |  |  |
| 8450 | 8449 |  |  |  |  |  |  |
|  |  |  |  |  | 8466 |  | 8470 |
|  | 8560 |  |  |  |  | 8563 | 8564 |
|  |  |  |  |  | 8559 |  |  |
|  |  |  |  |  |  |  | 8579 |
| 8592 | 8595 |  | 8592 | 8592 | 8593 | 8592 | 8592 |
| 8602 |  |  |  |  |  |  |  |
|  |  |  | 8607 |  | 8609 |  | 8607 |
|  |  |  |  |  |  |  | 8664 |
| 8749 |  | 8749 |  |  |  |  |  |
|  |  |  |  |  | 8759 |  |  |
|  |  |  |  |  |  | 8778 |  |
|  |  |  |  |  | 8789 |  |  |
| 8809 | 8806 | 8807 | 8806 | 8806 | 8806 | 8806 | 8806 |
|  |  |  |  |  | 8833 |  |  |
|  |  |  |  |  | 8868 |  |  |
|  |  |  |  |  | 8902 |  |  |
| 8910 | 8911 | 8911 | 8911 |  | 8911 |  |  |
|  |  |  |  | 8921 | 8923 | 8922 |  |
|  |  |  |  |  | 8959 |  |  |
|  | 8966 | 8966 | 8966 | 8966 | 8965 |  |  |
|  |  |  |  |  | 8981 | 8981 |  |
|  |  |  |  |  | 8997 | 8997 |  |
| 9029 | 9028 | 9030 |  |  | 9031 |  |  |
| 9074 | 9072 | 9072 | 9072 | 9071 | 9071 | 9073 | 9073 |
| 9094 |  |  |  |  |  |  | 9090 |
|  | 9115 |  | 9115 | 9113 | 9113 | 9113 | 9111 |
| 9171 | 9170 |  | 9169 |  | 9167 |  |  |
|  |  |  |  |  |  |  | 9216 |
|  |  |  | 9224 |  |  |  |  |
|  |  |  |  |  | 9245 | 9246 |  |
|  |  |  |  |  |  |  | 9252 |
|  | 9260 |  | 9259 |  | 9258 | 9259 | 9258 |
| 9276 |  |  |  |  |  |  | 9276 |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 9342 | 9337 | 9338 | 9336 | 9336 | 9335 | 9339 | |
| | 9352 | | 9354 | 9351 | 9353 | 9353 | 9351 |
| | | | | | | | 9406 |
| | 9435 | | | | | | |
| | | | | | | | 9464 |
| | | | | | | | 9507 |
| | | | | | 9522 | | |
| | 9527 | | | | | | |
| | | | 9534 | | | 9537 | 9534 |
| | | | | | | | 9550 |
| 9577 | 9578 | 9577 | 9577 | 9576 | 9576 | 9576 | 9576 |
| | | | | | 9594 | 9592 | 9592 |
| | | | | | | | 9642 |
| | | | | | | | 9718 |
| | | 9676 | | | 9674 | | |
| | | | | | 9680 | | |
| | | | | | | | 9806 |
| 9813 | | | | 9813 | 9812 | | |
| | | | | | | | 9876 |
| | | | | | | | 9904 |
| | | | | | | | 10000 |
| | | | | | 10026 | | |
| | | | | | | | 10038 |
| 10198 | 10193 | 10195 | 10194 | 10195 | 10194 | 10194 | 10200 |
| | | 10207 | | | | | 10213 |
| | | | | | | | 10227 |
| | | | | 10264 | | 10266 | 10265 |
| | | | | | | 10282 | |
| | | | | 10292 | 10292 | | |
| | | | | | | | 10304 |
| | | | | | 10310 | | |
| | | | | | | | 10315 |
| 10327 | | | | | | | |
| | | | | 10338 | 10335 | | 10332 |
| | | | | | 10358 | 10357 | 10354 |
| | | | | | | 10373 | |
| | | | 10403 | | | | |
| | | | | | | 10413 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 10420 | | | | | | | |
| | | | | | 10458 | 10457 | |
| | | | | | 10476 | 10477 | |
| | | | | | 10732 | | |
| | | | | | | | 10780 |
| | | | | | | | 10793 |
| 10829 | | | | 10826 | | | |
| | | | | 10836 | 10833 | | |
| | | | | 10851 | | | |
| | | | | | | 10864 | 10867 |
| 10894 | 10893 | 10895 | 10893 | 10893 | 10891 | 10894 | |
| 10925 | 10922 | 10922 | 10921 | | | | |
| 10955 | | | | | | | |
| 10980 | | | | | | | |
| | | | | 11069 | 11067 | 11066 | |
| | | | | | 11159 | | 11159 |
| | | | | | 11164 | | |
| | | | | 11184 | 11181 | | |
| | | | | | 11206 | | |
| | | | | 11240 | 11237 | 11240 | |
| | | | | | | 11248 | |
| 11256 | | | | | | | |
| | | | | 11273 | 11271 | 11270 | 11270 |
| | | | | | | 11291 | |
| | | | | | | | 11303 |
| | | | | | 11314 | | |
| 11398 | 11400 | 11398 | 11398 | 11399 | 11398 | 11396 | |
| | | | | | | 11416 | |
| | | | | | | 11437 | 11439 |
| 11450 | 11444 | | 11447 | 11446 | 11446 | 11448 | |
| | | | | 11464 | 11463 | 11464 | 11465 |
| | | 11479 | | | 11478 | 11478 | 11478 |
| | | | | | | | 11487 |
| | | | | | | 11493 | |
| | 11556 | 11557 | | | | | |
| | | | 11563 | 11562 | | | |
| | | | | 11571 | | | |
| 11692 | | | 11695 | 11691 | 11692 | | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| 11701 | | 11704 | | | | | |
| | | | | | | | 11767 |
| | | | | | 11787 | 11788 | 11787 |
| | | | | | | 11801 | |
| | | | | | | | 11936 |
| | | | 11999 | | | | 11992 |
| | | | | | 12182 | 12181 | 12180 |
| | | | | | 12222 | | |
| | | | | | | 12268 | |
| | | | | | | | 12284 |
| 12270 | 12272 | | 12272 | 12271 | 12270 | | |
| | | | | | 12305 | 12310 | 12311 |
| 12358 | | | | | | | |
| 12369 | | | | | | | |
| | | | | 12396 | | 12397 | 12399 |
| | | | | | 12341 | | |
| | | | | | 12398 | | |
| | | | | 12414 | 12415 | 12414 | 12413 |
| | | | | | | | 12639 |
| | | | | | 12649 | | |
| | | | | | 12750 | | |
| | | | | 12898 | 12896 | | |
| | | | | 13034 | 13033 | 13036 | |
| | | | | 13093 | 13091 | | 13100 |
| | | | | | 13108 | | |
| | | | | 13120 | 13122 | 13120 | |
| | | | | | 13135 | | |
| | | | | | | 13166 | 13170 |
| | | | | | | | 13182 |
| | | | | | 13226 | 13227 | |
| | | | | 13257 | | 13255 | |
| | | | | | 13263 | | |
| | | | | | | 13274 | |
| | | | | 13297 | 13297 | 13299 | |
| | | | | 13331 | 13334 | 13332 | |
| | | | | 13351 | | | |
| | | | | 13463 | | | |
| | | | | | | 13534 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | 14073 | 14070 | |
| | | | | 14105 | | | |
| | | | | | | | 14117 |
| | | | | 14202 | | | |
| | | | | | | | 14214 |
| | | | | | 14418 | 14418 | 14416 |
| | | | | | 14434 | | |
| | | | | | 14624 | | |
| | | | | | | 14632 | |
| | | | | 14694 | | | |
| 14830 | | | | | | | |
| | | | 14839 | 14842 | 14840 | | |
| 14846 | 14850 | | | 14847 | | | |
| | | | | 14858 | 14857 | 14859 | |
| | | | | | 14916 | | |
| | | | | | | | 15527 |
| | | | | | 15466 | | |
| | | | | | | 15479 | |
| | | | | 15547 | 15549 | 15550 | |
| | | | | | | | 15570 |
| | | | | | 16115 | | |
| 16230 | | | | | | | |
| | | | | | 16251 | | |
| | | | | | | | 16265 |
| | | | | 16273 | 16270 | 16272 | |
| | | | | | | | 16282 |
| | | | | 16469 | 16469 | 16466 | 16469 |
| | | | | 16986 | 16984 | | |
| 17212 | 17210 | | 17208 | 17212 | 17208 | 17212 | 17214 |
| | | | | | 17230 | 17231 | 17231 |
| | | | | | | | 17252 |
| | | | | | 17387 | 17391 | |
| | | | | | 17404 | | 17398 |
| | | | | 17417 | 17417 | | 17417 |
| | | | | | | 17427 | |
| | | | | | | 17996 | |
| | | | | | 18135 | 18133 | |
| 19308 | | | | 19305 | | 19304 | |

| 3 h | 5 h | 12 h | 24 h | 48 h | 96 h | 168 h | 336 h |
|---|---|---|---|---|---|---|---|
| | | | | | 19220 | | |
| | | | | | 19301 | | |
| | | | | 19313 | | | 19318 |
| | | | | | | | 19333 |
| | | | | | 20407 | 20410 | 20415 |
| | | | | | | 20428 | |
| | | | | | | | 20448 |
| | | | | | | 22138 | |
| | | | | 22315 | | | |
| | | | | | 22340 | | |
| | | | | | | | 22510 |
| | | | | | | 22522 | |
| | | | | | | 26551 | |
| | | | | | | 26573 | |
| | | | | | 26982 | | |
| | | | | | | 31287 | |
| | | | | | | | 31301 |
| | | | | | | 31516 | |
| | | | | | 32164 | 32165 | |

**Table A.10** [M+H]$^+$ (Da) from *Microbacterium saperdae* (ATCC 19272) extracts by LC/off-line MALDI MS.

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | | | 2070 | | | |
| | | | 2079 | | | | |
| | | | | | | | 2210 |
| | | | | | 2307 | | |
| | | | | | | | 2369 |
| | | | | | | | 2374 |
| | | | | | | | 2420 |
| | | | | | | 2435 | 2433 |
| | | | | | 2438 | | |
| | | | | | | 2446 | 2447 |
| | | | | | | | 2456 |
| | | | 2524 | 2523 | 2524 | 2524 | 2523 |
| | | | | | | | 2530 |
| | | 2535 | | | | | 2536 |
| | | | | | | | 2541 |
| | | | | | | | 2546 |
| | | | 2551 | 2549 | | | |
| | | | | | 2608 | 2609 | |
| | | | 2658 | | | 2658 | |
| | | | | | | | 2661 |
| | | | | | | | 2691 |
| | | 2748 | | | | | |
| | | | | | | | 2812 |
| | | | 2819 | 2817 | 2818 | | |
| | | | | | | 2822 | |
| | | | | | | | 2833 |
| | | | | 2835 | | | |
| | | 2857 | | 2856 | | | |
| | | 2875 | 2875 | 2874 | 2874 | 2876 | |
| 2878 | | | | | | | |
| | | | | 2888 | | | |
| | | | | | | 2956 | |
| | | | 2963 | 2965 | | | |
| | | | | 2980 | | | |
| | | | | 3003 | | 3003 | |
| | 3009 | | | | | | |
| | | | 3019 | 3017 | | 3020 | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | 3144 | | | | | 3146 |
| | | 3153 | | | | | |
| | | | 3169 | | | | |
| | | | | | 3181 | | |
| | | | | 3185 | | | |
| | | 3201 | 3203 | 3202 | | | 3203 |
| | | | | 3286 | | | |
| | | | | | | | 3317 |
| | | 3372 | | 3371 | | | |
| | | | | | | | 3434 |
| | | | | | | | 3443 |
| | | | 3456 | | | | |
| | | 3559 | | 3559 | | | |
| | 3584 | 3584 | 3585 | | | 3586 | |
| | | | 3610 | | 3610 | 3611 | |
| | | | | | | 3619 | |
| | | | | 3633 | 3632 | | |
| | | 3636 | | 3636 | 3636 | 3635 | |
| | | | | | | 3640 | |
| | | | | | 3658 | 3657 | |
| | | | | | | 3678 | |
| | | 3684 | | | | | |
| | | | | | | | 3687 |
| | | | | | | 3695 | |
| | | | | | | 3700 | |
| | | | | | | | 3720 |
| | | | | | | 3743 | |
| | | 3748 | | | | | |
| | | | | | | | 3801 |
| | | | | | | 3804 | |
| | | | | | | 3823 | |
| | | | | | 3882 | | |
| | | | | | | 3901 | |
| | | | | 3907 | | | |
| | | | | 3933 | 3934 | 3934 | |
| | | | | | | 3948 | |
| | | | | | | 3970 | |
| | | | 3974 | | | 3975 | 3973 |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | | | | | | 3990 |
| | | | | | 4024 | | |
| | | 4033 | | | | | |
| | | 4052 | | | | | |
| | | | | | | | 4066 |
| | | | | | | 4086 | |
| | | 4164 | 4167 | 4165 | | | |
| | | 4206 | | | | | |
| | | | | | | | 4220 |
| | | | | | | | 4267 |
| | | | 4270 | 4271 | | 4272 | |
| | | | | | 4292 | | |
| | | | 4319 | | 4318 | 4318 | |
| | | | | | | 4340 | |
| | | | | 4345 | | | |
| | | | 4348 | 4349 | | | |
| | | | 4365 | | | | |
| | | 4369 | | 4369 | 4368 | | |
| | | | | | 4388 | | |
| | | | | | 4427 | 4428 | |
| | | | 4431 | | | | |
| | | | | | 4439 | | |
| | | | | | | 4452 | |
| | | | | | | | 4469 |
| | | | | | | 4474 | |
| | | | | | | | 4490 |
| 4547 | | 4545 | | 4545 | | | |
| | | | | | 4557 | 4558 | |
| | | | | | | 4562 | |
| | | 4587 | 4587 | 4587 | 4587 | 4588 | |
| | | | | | | 4596 | |
| | | | | | | 4609 | |
| | | | | 4637 | 4637 | | |
| | | | | | | 4684 | |
| | | | | | | | 4698 |
| 4709 | 4710 | 4710 | 4712 | 4710 | 4710 | | |
| | | 4723 | | | | | |
| | | | 4733 | | | | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | | | 4745 | 4746 | | |
| | | | 4767 | 4765 | | | |
| | 4770 | | | | | | |
| | | 4869 | | | 4867 | 4869 | |
| | | | | | | 4874 | |
| | | 4896 | | | | | |
| | | | | | 4908 | | |
| | | | | | | 4987 | |
| | | | 4992 | 4990 | | | |
| | 5007 | | | | | | |
| | | | | | | 5028 | |
| | | | | | 5046 | | |
| | | | | | | 5049 | |
| | | | | 5060 | | | |
| | | | | | | | 5074 |
| | | | | 5078 | | | |
| | | | 5092 | 5091 | | | |
| | | | | | 5096 | 5097 | |
| | | 5101 | 5101 | 5101 | | 5102 | |
| | 5109 | 5110 | 5110 | | 5109 | 5110 | 5109 |
| | | 5128 | | | | | |
| 5133 | | | | | | 5131 | |
| | | 5149 | | | | | |
| | | 5153 | 5153 | 5151 | 5152 | 5153 | |
| | | | | | 5174 | | |
| | | | | | | 5183 | |
| | | | | 5189 | | | |
| | | | | | | 5210 | |
| | | | | 5220 | | | |
| | | | | | 5225 | | |
| | | | | | | | 5350 |
| | | | | | | | 5420 |
| | | | | | | | 5505 |
| | | | | | | | 5515 |
| | 5523 | | | | | | 5522 |
| 5537 | 5535 | | | | | 5538 | |
| | | 5594 | | | | | |
| | | | | | 5643 | 5644 | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | 5739 | | | | | |
| | | | 5801 | | | 5799 | |
| | | | | | | | 5812 |
| | | | | | | | 5846 |
| | 5880 | | | | | | |
| | | | | 5897 | 5896 | | |
| | | | | | | 5904 | |
| | | | | 5930 | | 5932 | |
| | | | | 5944 | | | |
| | | | | | | 5956 | |
| | | | | | | 5974 | |
| | | 5997 | 5998 | 5997 | 5997 | 5998 | |
| | | | | 6023 | | | |
| | | | | | | 6052 | |
| | 6098 | 6097 | 6098 | | | | |
| | | 6115 | | | | | |
| | | | | | | | 6125 |
| | | | | 6184 | 6184 | 6184 | |
| | | 6200 | | | | | |
| | | | | | | | 6224 |
| | | 6289 | | 6289 | 6290 | 6290 | |
| | | 6303 | 6301 | | 6304 | 6303 | |
| | | | | | | | 6344 |
| | | | | | | | 6391 |
| | | | 6411 | 6409 | | | |
| | | | | | | | 6419 |
| | | | | | | 6440 | 6440 |
| | | | | | | 6475 | |
| | | | | 6524 | 6526 | | |
| | 6544 | 6541 | 6544 | | 6542 | 6543 | |
| | | | | | | | 6576 |
| | 6601 | | | | | | |
| | | | 6667 | | | | |
| 6674 | | 6672 | | | | | |
| | | 6751 | 6751 | | | | |
| | 6758 | | | | 6755 | | |
| | | | | | | 6761 | |
| | | | | | | 6829 | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| | | | | | | | 6860 |
| | | | | | | 6901 | |
| | | | | | | 6994 | |
| 7042 | | 7038 | | 7041 | | 7044 | 7044 |
| | | | | | | 7066 | |
| | | | | 7081 | | 7082 | |
| 7145 | | | | | | | |
| | | | 7152 | | 7155 | | |
| 7164 | | | | | | | |
| | | | | | 7169 | | |
| 7181 | 7181 | 7180 | 7180 | 7180 | 7180 | | |
| | 7196 | | 7193 | | 7196 | 7195 | 7195 |
| | | 7200 | | | | | |
| | | | 7207 | | | | |
| | 7238 | | | | | | |
| | 7257 | | | | | | |
| | | | 7296 | | | | |
| 7316 | 7315 | 7317 | 7314 | 7316 | 7316 | 7316 | |
| | | | | | 7321 | | |
| 7338 | 7337 | 7338 | 7338 | 7338 | 7337 | 7338 | 7339 |
| | | | | | 7351 | 7352 | |
| | | 7357 | 7355 | | 7359 | | |
| | 7370 | | | 7367 | 7368 | | |
| | 7395 | | | | 7395 | | |
| 7468 | 7468 | | | | | | |
| | | 7534 | 7533 | 7534 | 7530 | | |
| | | | | | | 7543 | |
| | 7551 | 7551 | 7552 | 7553 | 7552 | | |
| | | 7568 | | | 7568 | | |
| 7578 | 7577 | 7578 | | | 7577 | 7579 | 7579 |
| | | | | | | | 7635 |
| 7873 | | | 7872 | 7874 | 7875 | 7875 | |
| | | 7899 | | | | | |
| | | | | | | 7924 | |
| | | | | | | | 8236 |
| | | | | | | | 8248 |
| | | | | | | | 8268 |
| | 8378 | | | | | | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| 8425 | | | | | | | |
| 8692 | 8692 | 8694 | | | 8693 | | 8695 |
| 9019 | 9020 | 9019 | 9021 | | | 9024 | |
| | 9038 | 9036 | | | | | |
| 9092 | | | | | | | |
| | | 9184 | | | | | |
| | | | | | | | 9396 |
| 9528 | 9528 | 9528 | 9529 | 9528 | 9529 | 9529 | 9526 |
| | | | 9550 | | | | |
| | 9560 | | | | | | |
| | 9585 | | | | | | |
| | 9590 | | | | | | |
| | | | | | | | 9638 |
| 10005 | 10006 | 10006 | 10005 | 10006 | 10008 | 10005 | |
| | | 10024 | | | 10021 | | |
| | | | | | | 10326 | |
| 10361 | 10362 | 10363 | 10363 | 10361 | 10361 | | |
| | | | 10384 | | | | |
| | | | 10396 | | 10395 | | |
| | | 10405 | | | | | |
| | | | 10410 | | | | |
| 10421 | 10421 | | | | | | |
| 10461 | | | | | | | |
| | | | | 10494 | | 10499 | |
| | | | | | | 10539 | |
| 10550 | | | | | | | |
| | 10607 | | | | | | |
| | | | | | | | 10837 |
| | | | | | | | 11239 |
| | | | | | | | 11367 |
| | 11377 | 11379 | | | 11379 | 11379 | |
| | 11475 | | | | | | |
| 11646 | 11645 | 11648 | 11644 | | 11646 | | |
| | | 11685 | | | | | |
| 11794 | 11789 | | | | | | |
| 12603 | 12604 | 12602 | | | | | 12601 |
| 12741 | | | | | | | |
| | 12748 | 12747 | | | | | |

| 18 h | 20 h | 24 h | 32 h | 48 h | 72 h | 144 h | 360 h |
|---|---|---|---|---|---|---|---|
| 12951 | 12949 | 12948 | | | 12949 | | |
| 13610 | | | | | | | |
| | | | | | | | 13895 |
| 14308 | 14304 | 14305 | | | 14311 | | |
| 15447 | | 15448 | | | | | |
| 17103 | 17101 | 17111 | | | | | |

**Table A.11** $[M+H]^+$ (Da) from *E. coli* (ATCC 9637) extracts of the second batch by LC/ESI-MS*.

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2097.4 | | | | | | | | | | |
| | | | | | | | | 2244.1 | | | |
| | | | | | | | 2246.8 | 2246.6 | | | |
| | | | | | | | | 2250.3 | | | |
| | | | | | | | | | **2389.1** | **2388.8** | **2388.8** |
| | | | | | | | | 2390.1 | | | |
| **2430.6** | | | | | | **2431.0** | **2430.9** | | **2430.8** | **2430.7** | **2430.8** |
| | | | | | | | | 2432.9 | | | |
| | | | | | | | | | | | **2444.6** |
| | | | | | | | **2453.8** | | **2453.9** | **2453.1** | **2453.2** |
| | | | | | | | | 2455.3 | | | |
| | | | | | | **2473.0** | **2472.3** | | **2472.8** | | **2472.8** |
| | | | | | | | | 2474.6 | | | 2474.2 |
| 2570.5 | | | | | 2570.2 | | | | | | |
| 2618.4 | | | | | | | | | | | |
| | | | | | | | | 2643.8 | | | |
| | | | | | | | | 2721.2 | | | |
| | | | | 2802.9 | | | | | | | |
| | | | | | | | | | | | 2835.5 |
| 2942.4 | | | | | | | | | | | |
| | | | | 2997.6 | | | | | | | |
| 3039.2 | | | | | | | | | | | |
| | 3083.5 | | | | | | | | | | |
| | | | | | | | | 3243.9 | | | |
| **3412.3** | | **3412.6** | **3412.7** | **3412.0** | **3412.6** | | | | | | |
| | | | | 3651.8 | | | | | | | |
| | | | | | | | | 3703.3 | | | |
| | | | | | | | 3933.2 | | | | |
| | | | | | | | | 4091.5 | | | |
| | | | | | | | | 4196.9 | | | |
| | | | | 4106.4 | | | | | | | |
| **4332.8** | **4333.5** | **4331.9** | **4331.7** | **4332.5** | **4332.6** | | **4333.7** | | | | |
| **4363.1** | **4363.9** | | | | **4364.0** | | | **4364.0** | | | |
| 4425.0 | | | | | | | | | | | |
| | | | | 4426.5 | | | | | | | |
| 4427.8 | | | | | | | | | | | |
| **4431.2** | **4431.8** | **4431.7** | **4431.1** | **4431.1** | **4432.2** | | | | | | |
| 4459.9 | 4462.9 | | | | | | | | | | |
| | | | | | 4517.2 | | | | | | |
| 4524.4 | | | | | | | | | | | |
| | | | | | | | | 4529.4 | | | |
| | | | | 4553.6 | | | | | | | |
| | | | | | | | 4614.6 | | | | |
| | | | **4648.0** | | **4648.5** | | | | | | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 4713.5 | | | | | | | |
| | | | | 4719.9 | | | | | | | |
| | | | | | | | | 4725.8 | | | |
| | | | | | | | | 4773.4 | | | |
| 4787.4 | | | | | | | | | | | |
| | | | | 5055.3 | | | | | | | |
| | | | | | | | | 5064.8 | | | |
| | | | | 5075.6 | 5075.0 | | | | | | |
| | | | | | **5095.5** | **5097.1** | | **5098.3** | | | |
| | **5171.0** | **5171.0** | **5170.8** | **5171.0** | **5171.0** | | **5170.7** | | | | |
| | | 5247.0 | | | | | | | | | |
| | | | | | | 5261.7 | | | | | |
| | **5268.9** | | | | **5270.0** | | | | | | |
| | | | | 5298.1 | | | | | | | |
| | | | | 5300.3 | | | | | | | |
| | | | | 5308.3 | | | | | | | |
| | | | | | | | | 5326.5 | | | |
| | | | | | 5331.7 | | | | | | |
| | | | 5432.0 | 5431.5 | 5431.4 | | | | | | |
| | | | | **5441.1** | **5441.2** | | | | | | |
| | | | | | 5462.5 | | | | | | |
| | | | | | | | | 5464.7 | | | |
| | | | | | | 5477.2 | | | | | |
| | | | | | 5539.8 | | | | | | |
| 5547.2 | | 5547.3 | | | | | | | | | |
| | 5560.8 | | | | | | | | | | |
| | | | | | | | | 5616.2 | | | |
| 5715.9 | | | | | 5717.1 | | | | | | |
| | | | | | | | | | 5751.1 | | |
| | | | | | | | | | 5881.3 | | |
| **5884.8** | **5885.9** | **5883.5** | **5884.1** | **5884.7** | **5884.6** | **5884.6** | | | | | |
| | | | | | | 5888.5 | | | | | |
| | **5916.1** | | | | | | | | | | |
| | | | | | | | | **5946.0** | | | |
| | | | | 5961.9 | | | | | | | |
| | | | | | 5973.5 | | | | | | |
| | | | | | | | | 6065.9 | | | |
| | 6080.7 | | | | | | | | | | |
| | | | | **6087.3** | | | | | | | |
| | | | | 6139.3 | | | | | | | |
| | | | | | | | | 6157.4 | | | |
| | | | | | | | | 6170.0 | | | |
| **6260.4** | **6260.4** | **6258.6** | **6261.0** | **6260.2** | **6259.6** | **6262.2** | **6258.5** | | | | |
| | | 6266.6 | | | | | | | | | |
| | | **6271.0** | | | | | **6271.6** | **6269.7** | | | |
| | 6277.4 | | | | | | | | | | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | **6290.8** |  |  |  |  |  |  |  |  |  | **6289.7** |
|  |  |  |  | **6313.4** | **6315.7** | **6315.5** | **6313.5** | **6314.5** |  |  |  |
|  |  |  | 6317.3 |  |  |  |  |  |  |  |  |
|  |  | **6324.2** |  |  |  |  |  |  | **6323.0** | **6323.1** |  |
|  |  |  |  |  |  |  | 6325.6 |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  | 6345.0 |
|  | **6357.4** |  |  | **6357.6** |  |  |  |  |  |  |  |
| 6359.5 | 6359.4 |  |  | 6362.9 |  |  |  |  |  |  |  |
| 6361.1 |  |  |  |  |  |  |  |  |  |  |  |
|  | 6383.2 |  |  | 6382.6 | 6381.0 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 6413.5 | 6411.3 | 6411.8 |  |
| **6425.1** | **6425.4** | **6425.1** | **6425.5** | **6425.3** | **6425.5** | **6425.2** | **6425.1** | **6426.7** | **6424.4** |  | **6427.0** |
|  | 6447.6 |  |  |  | 6446.9 |  |  |  |  |  |  |
|  | 6462.2 |  |  |  |  |  |  |  |  |  |  |
| 6480.0 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  | 6520.9 | 6521.3 | 6521.1 |  |  |  |  |  |  |
| **6524.7** |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 6549.7 |  |  |  |  |  |  |
| 6556.2 | 6556.7 | 6556.4 |  |  |  | 6557.4 |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  | 6578.0 |
|  | 6599.6 |  |  |  | 6599.4 |  |  |  |  |  |  |
| **6609.9** | **6610.3** | **6609.4** | **6610.6** | **6609.9** | **6610.1** | **6609.8** | **6610.7** |  |  |  |  |
|  |  | **6659.6** |  |  | **6656.5** |  |  |  |  |  |  |
|  |  |  |  |  | 6667.9 |  |  |  |  |  |  |
|  | **6670.2** |  | **6670.4** | **6670.1** | **6670.7** |  |  |  |  |  |  |
|  |  |  | **6683.7** |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  | **6694.6** |  |  |
|  |  |  |  |  |  |  |  |  |  | **6709.0** |  |
|  |  |  |  |  |  |  |  |  |  | **6766.0** |  |
|  |  |  |  |  |  |  |  | **6781.1** |  |  | **6781.8** |
|  |  | 6789.6 |  |  |  |  |  |  |  |  |  |
| **6792.6** | **6792.8** | **6792.5** | **6792.9** | **6792.8** | **6792.8** | **6792.6** | **6791.8** |  |  |  |  |
|  |  |  |  |  |  | 6802.8 |  |  |  |  |  |
|  |  |  |  |  |  | 6811.5 |  |  |  |  |  |
| 6852.6 |  |  |  |  |  |  | 6854.6 |  |  |  |  |
| 6857.1 | 6858.1 |  |  |  |  |  |  | 6857.2 | 6856.3 |  | 6856.3 |
| **6859.6** | **6891.1** |  |  | **6891.7** | **6891.5** |  |  |  |  |  |  |
| 6872.6 |  |  |  |  |  |  |  |  |  |  |  |
| 6890.7 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 6912.8 |  |  |  |  |  |  |
| 7035.1 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 7073.5 |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  | **7082.0** |  |  |
|  |  |  |  |  |  |  |  |  | 7140.7 | 7140.1 |  |
|  |  |  | **7161.3** | **7161.9** | **7161.7** |  | **7162.8** |  |  |  |  |
|  |  | **7178.5** | **7180.1** | **7178.7** | **7178.8** |  | **7178.3** |  |  |  |  |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **7212.7** | | | | | | | | | | | |
| **7239.3** | | **7239.8** | **7239.8** | **7239.6** | **7239.5** | | | | | | |
| | | | | | 7243.9 | | | | | | |
| | | | | | | **7263.7** | **7261.7** | | | | |
| | | 7267.3 | | | | | 7270.8 | | | | |
| **7274.0** | | | | | **7274.7** | | **7273.5** | | **7274.3** | | **7273.5** |
| | | | | | | | 7277.6 | | 7280.4 | | |
| 7300.2 | 7300.7 | 7299.9 | | | | | | | | | |
| | | | | | **7320.2** | | | | **7319.5** | | |
| | | | | | | | 7323.4 | | | | |
| **7329.8** | **7329.0** | | | | **7332.3** | **7332.4** | | | **7332.4** | | |
| 7358.9 | | | | | 7357.6 | | | | | | |
| **7367.1** | **7366.5** | **7366.6** | **7366.1** | **7366.8** | **7366.6** | **7366.3** | **7365.8** | **7367.5** | **7366.3** | **7363.6** | **7366.7** |
| | | | | | | 7376.6 | | 7375.6 | | | |
| | | 7388.5 | 7390.1 | | 7387.9 | | | | | | |
| **7394.3** | **7394.4** | **7394.5** | | | | | | | | | |
| | | | | 7403.3 | | | | | | | |
| | 7409.8 | | | | | | | | | | |
| | | 7413.8 | | | | | | | | | |
| | 7416.9 | | | 7418.1 | | | | | | | |
| | | | | | | | | | | | 7436.6 |
| | | | | | | | 7520.8 | | | | |
| | | | | | | | | 7627.4 | | | |
| | | | | | | | 7637.8 | | | | |
| | | | | | | **7706.9** | | **7708.4** | **7707.1** | **7706.9** | |
| | | | | | | | | | | 7713.1 | |
| | | | **7732.5** | **7732.5** | **7732.7** | **7733.0** | **7732.6** | **7734.1** | | | |
| 7752.4 | | | | | | | | | | | |
| | | | | | | 7761.1 | | | | | |
| **7768.2** | **7768.6** | **7768.6** | **7768.9** | **7768.8** | **7768.8** | **7769.2** | **7769.1** | | | | |
| | | 7773.9 | | | 7776.6 | 7773.7 | | | | | |
| | | | | | | | | | 7793.6 | | |
| | **7796.8** | | **7798.0** | | **7795.4** | **7796.8** | | | | | |
| 7834.9 | 7834.4 | 7834.5 | 7835.6 | 7834.6 | 7834.8 | 7836.3 | | | | | |
| | | | | 7865.1 | 7866.3 | | | | | | |
| | **7867.4** | | **7867.1** | | | | | **7866.6** | **7868.0** | | |
| 8055.1 | 8055.1 | | 8054.8 | | 8055.7 | | | | | | |
| | | | | | **8068.1** | | **8068.9** | | | | |
| | | | 8119.4 | | | | | | | | |
| **8138.8** | | | | | | | | | **8138.0** | **8137.5** | **8137.1** |
| | | | | | | | | 8176.5 | | | |
| | | | | | | | | | | | 8214.2 |
| | | | | 8301.5 | | | | | | | |
| | | | | | | | **8328.0** | **8325.4** | **8326.0** | **8325.8** | **8326.0** |
| | | | | | | | | | | 8391.7 | |
| | | | | | 8454.5 | | | | | | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 8487.4 | | | 8486.9 | | |
| | | | | | | | | | 8502.1 | | |
| | | | | | | | | | | 8529.1 | |
| | | | | | | | | 8538.1 | | | |
| | | | | | | | | | **8546.1** | **8547.0** | **8546.4** |
| | | | | | | | | | 8551.4 | | |
| | | | | | | | | | | 8558.3 | |
| | | | | | | | | | **8574.8** | **8576.3** | |
| | | | | | | | | | 8591.0 | | |
| | | | | | | | | | 8593.0 | 8592.2 | 8593.6 |
| | | | | 8605.3 | | | | | | 8608.8 | |
| | | | **8682.1** | **8682.4** | **8683.2** | **8682.5** | | | | | |
| | | 8722.8 | | | | 8721.1 | | | 8721.0 | | 8721.5 |
| | **8726.6** | **8726.5** | **8726.2** | **8726.8** | **8726.5** | **8726.4** | **8726.0** | | **8728.1** | | |
| | | | | | | | | | | | 8738.2 |
| | | 8766.3 | | | | | | 8766.7 | | | |
| | | | | | **8781.4** | **8780.8** | | | | | |
| | | | | | | 8785.4 | | | | | |
| 8824.4 | 8824.5 | | | | | | | 8824.1 | | 8824.5 | |
| | | | | **8869.5** | | **8869.8** | | | | | |
| | | | | | | | 8855.2 | 8855.1 | | | |
| **8878.9** | **8879.7** | **8878.8** | | **8877.7** | | **8875.4** | | | | | |
| | | | | | | | **8899.9** | **8900.3** | **8897.1** | | |
| | | | | | | | | 8913.4 | | | |
| 8922.9 | | | | | | | | | | | |
| 8953.5 | 8953.6 | | | | | | | | | | |
| | | 8965.1 | | | | | | | | | |
| | | | | | | | | | | | 8990.1 |
| | | | | | | **8993.9** | | | | | |
| | | | | | 8897.5 | | | | | | |
| 9017.0 | | | | | | | | | | | |
| | | | | | | | | | | | 9052.3 |
| | | | | | **9064.8** | **9063.9** | **9063.6** | **9064.8** | **9063.9** | **9063.6** | **9064.3** |
| | | | | | | | | | 9084.9 | 9085.7 | |
| | | | | | | | | | 9119.5 | 9120.2 | 9120.5 |
| 9137.5 | 9137.9 | | | | | | | | | | |
| | | | | | 9167.9 | | | | | | |
| 9181.9 | | | | 9182.3 | | | | | | | |
| | | | | **9190.8** | **9191.5** | **9192.1** | | | **9191.2** | **9191.1** | **9191.7** |
| | | | | | **9195.4** | | | | | | **9192.2** |
| | | | | | | | | | | 9200.7 | |
| **9210.8** | **9212.4** | **9211.6** | **9212.2** | **9211.7** | **9212.4** | **9212.3** | **9212.1** | **9212.6** | | | |
| | | | | | | | 9215.4 | | | | |
| **9225.9** | **9228.3** | | **9227.5** | **9227.6** | | **9226.1** | **9225.9** | | **9226.3** | **9226.4** | **9226.4** |
| | | | | | 9229.8 | | | | | | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9232.9 | 9233.8 | | 9233.6 | | 9233.8 | | 9233.6 | | | | |
| | | 9237.3 | | 9237.3 | | | | | | | |
| 9254.6 | | | | | | | | | | | |
| | | | | | | | | | **9272.1** | | |
| | | | | | | 9283.5 | | | | | |
| | | | | | | 9293.1 | | | | | |
| | | | | | | | | | | | 9306.2 |
| **9309.4** | **9311.9** | | | **9309.4** | **9311.0** | | | | | | |
| | | | **9324.5** | | | | | | **9321.9** | **9323.1** | **9320.9** |
| **9341.0** | **9341.5** | **9340.8** | **9342.7** | **9341.1** | **9341.3** | **9341.0** | **9339.8** | **9339.6** | | | |
| | | 9344.2 | | | | | | 9345.4 | | | |
| | | | 9364.2 | | | | | | | | |
| | | | **9404.5** | | | | | | | | |
| | | | | | | | | | | | 9433.6 |
| **9439.5** | **9439.7** | | | **9439.7** | **9440.6** | | | | | | |
| | | | | | | | | 9472.2 | | | |
| | | | | | | | 9476.6 | | | | |
| **9525.3** | **9525.5** | **9526.3** | | | **9525.6** | | **9526.8** | | | | |
| | | | | **9532.0** | **9528.7** | | | | | | |
| **9535.2** | **9538.8** | | | | | **9535.8** | **9535.2** | **9536.4** | **9536.2** | **9535.7** | **9535.0** |
| 9548.0 | | | | | 9549.1 | | | 9550.1 | | | |
| | | | | | | | **9553.3** | | | **9553.5** | |
| | | | | | | | **9557.9** | **9557.9** | | | |
| | | | | | | | | | | | 9564.5 |
| | | | | | | | | | | | 9582.4 |
| **9592.1** | | | | | **9592.6** | | **9592.4** | **9593.4** | | | **9591.1** |
| | | | | | | | | | | 9617.4 | |
| **9624.2** | **9624.0** | | | | | | | | | | |
| **9642.6** | **9643.0** | **9642.7** | **9642.9** | **9642.6** | **9643.0** | **9643.0** | **9642.6** | **9643.7** | **9643.1** | | **9641.7** |
| 9665.1 | | | | 9663.6 | | | | | | | |
| | **9670.5** | **9671.1** | **9670.9** | **9670.2** | | | **9669.8** | | | | |
| | | | | | | | 9681.4 | | | | |
| | | | | | | | 9685.3 | | | | |
| | | | | | | | | | 9721.4 | 9721.0 | 9721.2 |
| **9729.1** | **9730.1** | | **9731.0** | **9730.0** | **9728.3** | | **9729.6** | | | | |
| | | | | | | | | 9736.9 | | | |
| **9739.8** | **9739.6** | | **9742.0** | **9740.3** | **9740.6** | **9739.6** | **9739.7** | **9739.9** | **9740.2** | **9739.5** | **9740.4** |
| | | | | | | | | | | 9755.2 | 9755.3 |
| | | | | | | | | 9756.0 | 9756.6 | | |
| | | | | | | **9762.1** | **9762.6** | **9760.7** | **9762.1** | **9761.9** | **9762.1** |
| | | | | | | | **9772.1** | | | | |
| | | | | | | | | | 9777.8 | | 9779.2 |
| | | | | | | | | | 9784.0 | 9782.3 | |
| | | | | | | | | 9790.4 | 9790.2 | 9791.4 | 9793.0 |
| | | | | | | **9803.2** | | **9803.2** | **9802.8** | **9802.7** | **9803.7** |
| | | | | | | | | | 9827.1 | 9825.0 | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
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|  |  |  |  |  |  |  | **9836.4** |  | **9838.9** | **9839.5** | **9839.7** |
|  |  |  |  |  |  |  | **9857.2** |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 9864.8 |  |
|  |  |  |  |  |  |  |  |  | 9870.2 |  |  |
|  |  |  |  |  | **9901.0** |  | **9900.9** | **9901.1** |  |  |  |
|  |  | 9969.0 | 9967.2 |  |  |  |  |  |  |  |  |
| **9987.1** | **9987.4** | **9988.4** | **9987.3** | **9987.3** | **9987.4** | **9987.3** | **9986.2** | **9987.9** |  |  |  |
| 10008 | 10009 |  | 10009 | 10005 | 10008 |  |  |  |  |  |  |
|  |  |  | 10029 |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 10039 |  |  |  |  |
|  |  |  |  |  |  | **10058** | **10060** | **10059** |  |  |  |
| **10085** | **10086** | **10086** | **10086** | **10085** | **10087** |  |  |  |  |  |  |
| 10123 | 10126 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 10139 |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 10181 |  |
|  | 10204 |  |  |  |  |  |  |  |  |  |  |
|  |  | 10217 |  |  | 10217 |  | 10215 | 10217 | 10215 |  |  |
|  |  |  |  |  |  |  |  |  |  |  | 10247 |
|  |  |  |  |  |  |  | **10298** | **10296** |  |  |  |
|  |  |  |  |  |  |  | **10385** | **10385** | **10385** | **10385** | **10386** |
| **10431** | **10431** |  | **10429** | **10431** | **10430** | **10429** | **10428** |  |  |  |  |
| **10465** |  |  |  |  |  |  | **10467** |  | **10465** | **10464** | **10467** |
| **10497** | **10497** | **10497** | **10497** | **10497** | **10496** | **10497** | **10498** | **10497** |  |  |  |
|  | 10513 |  |  |  |  |  |  |  |  |  |  |
|  | 10519 |  |  |  | 10519 |  |  |  |  |  |  |
|  |  |  |  |  |  | 10538 |  |  |  |  |  |
|  |  |  |  |  |  |  | 10577 |  |  |  |  |
| **10595** |  |  |  |  | **10595** |  |  |  |  |  |  |
| **10650** |  | **10654** |  |  |  |  |  |  | **10651** | **10651** | **10650** |
| **10693** |  |  |  |  |  | **10694** |  | **10694** | **10693** | **10692** |  |
|  |  |  |  |  |  |  |  |  |  |  | 10696 |
|  | 10733 |  |  |  |  | 10732 |  |  |  |  |  |
|  |  |  |  |  |  | **10751** |  |  |  | **10751** |  |
|  |  |  |  |  |  |  |  | 10809 |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 10921 |  |
|  |  |  | **10935** | **10934** | **10935** | **10935** |  |  |  |  |  |
|  |  |  |  |  | 11034 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 11051 |  |
| 11116 |  |  |  |  |  | 11115 | 11115 |  |  |  |  |
|  |  | **11120** |  |  | **11120** |  |  |  |  |  |  |
|  |  |  |  |  | **11185** | **11185** |  | **11187** |  |  | **11186** |
|  | 11190 | 11190 |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 11223 | 11223 |  | 11223 | 11223 | 11224 |
| **11229** | **11229** | **11228** | **11229** | **11229** | **11229** | **11228** | **11229** | **11227** |  |  |  |
|  |  |  |  |  |  |  | 11231 |  |  |  |  |
| **11250** |  |  |  | **11250** |  |  |  |  |  |  |  |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
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|  |  |  |  | 11268 |  | 11270 |  |  |  | 11270 |  |
|  |  |  |  |  | **11291** |  |  |  |  |  |  |
|  |  |  |  | **11329** |  |  |  |  |  |  |  |
| **11345** | **11345** | **11345** | **11344** | **11345** | **11346** | **11345** | **11346** | **11345** |  |  |  |
|  |  |  |  |  |  | **11450** |  | **11451** |  |  |  |
| **11554** | **11553** |  | **11554** | **11553** | **11554** | **11553** | **11553** | **11554** |  |  | **11551** |
|  |  |  |  |  | 11576 |  |  |  |  |  |  |
|  |  |  |  |  |  | 11686 |  |  |  |  |  |
|  | 11724 |  |  |  |  |  |  |  |  |  |  |
|  | 11780 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 11813 |  |  |  |
|  |  |  |  | 11827 |  |  |  |  |  |  |  |
|  |  |  |  |  |  | **11977** | **11976** |  | **11977** | **11977** |  |
|  |  |  |  | 12142 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 12167 |  |  |  |
|  |  |  |  |  |  |  | 12207 | 12208 | 12205 | 12206 | 12208 |
|  |  |  |  |  |  |  |  |  |  |  | **12227** |
|  |  |  |  |  |  |  |  |  |  | 12272 |  |
|  |  |  |  |  |  |  |  |  | 12318 | 12318 | 12318 |
|  |  |  |  |  |  |  | 12370 |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 12416 |  |
|  |  |  |  |  |  | 12472 |  |  |  |  |  |
|  | **12518** | **12517** | **12519** | **12518** |  |  |  | **12519** | **12517** |  |  |
| **12535** | **12536** | **12534** | **12536** | **12536** | **12536** | **12537** | **12535** | **12537** | **12537** | **12535** | **12536** |
|  | **12545** |  | **12544** |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  | 12608 |
| **12633** |  |  |  | **12636** |  | **12634** |  | **12634** |  |  |  |
|  |  |  |  |  | 12653 | 12653 | 12653 |  |  |  | 12653 |
|  | 12732 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | **12769** |  |  |  |  |  |
|  | 12913 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 12970 | 12973 |  |  |  |  |
|  |  |  |  |  |  | 13002 |  |  | 13004 |  |  |
| **13106** | **13106** |  | **13106** | **13106** | **13106** | **13107** |  |  |  |  |  |
| 13120 | 13120 |  | 13121 | 13121 | 13121 | 13121 | 13120 | 13123 |  | 13119 |  |
|  |  |  |  |  |  |  |  |  |  | 13126 |  |
|  |  |  |  |  |  | **13167** |  |  |  |  |  |
|  | **13205** |  |  |  |  |  |  |  |  |  |  |
|  | 13218 |  | 13220 |  |  |  |  |  |  |  |  |
|  |  |  | **13319** | **13319** | **13320** | **13318** | **13319** | **13221** |  |  |  |
|  |  |  | 13418 |  |  |  |  |  |  |  |  |
|  | 13433 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | **13481** | **13481** |  |  |  |  |  |
| **13623** | **13624** | **13622** | **13624** | **13623** | **13623** |  | **13624** | **13624** |  |  |  |
|  | **13649** |  | **13651** |  |  | **13651** |  |  |  |  |  |
|  |  |  |  |  |  | 13662 |  |  |  |  |  |
|  |  |  |  |  | 13675 |  |  |  |  |  |  |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
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|  | **13722** |  |  | **13722** | **13722** | **13721** |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 13738 |  |  |  |
|  | 13743 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  | 13775 |  |
| 13815 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  | 13956 |  |  |  |
|  |  |  |  |  |  |  |  | 14096 |  |  |  |
| 14112 | 14113 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  | 14147 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  | 14281 |  |  |
|  |  |  |  |  |  |  | 14284 | 14288 |  |  |  |
|  |  |  |  |  |  |  | 14330 |  |  |  |  |
| **14360** | **14360** |  | **14360** | **14360** | **14360** | **14361** |  |  |  |  |  |
|  |  |  |  | 14433 |  |  |  |  |  |  |  |
| **14457** | **14458** |  |  | **14458** | **14459** |  |  |  |  |  |  |
|  | 14461 |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  | 14628 |  |  |  |  |  |  |  |
|  |  | 14634 |  |  |  |  |  |  |  |  |  |
|  | **14752** |  | **14753** |  |  | **14752** | **14752** |  |  |  |  |
|  |  |  |  |  | 14871 |  |  |  |  |  |  |
| 14887 |  |  |  |  |  |  |  |  |  |  |  |
|  |  | 14946 |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 14967 |  |  |  |  |  |  |
|  |  |  |  | 14952 |  |  |  |  |  |  |  |
| **14970** | **14971** |  | **14971** | **14971** |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  | 15176 |
|  |  |  |  |  |  | 15293 |  |  |  |  |  |
| **15315** | **15315** | **15315** | **15317** | **15315** | **15316** | **15316** | **15315** |  |  |  |  |
|  |  |  |  |  |  |  |  |  | 15409 |  | 15408 |
|  |  |  |  |  | 15337 |  |  |  |  |  |  |
|  | **15413** |  |  |  | **15412** |  |  |  |  |  |  |
|  |  |  |  | 15418 |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 15429 |  |  |  |  |
| **15491** | **15493** |  | **15492** | **15491** | **15493** | **15494** | **15492** | **15492** |  |  |  |
|  |  |  |  |  |  |  |  | 15516 |  |  |  |
| 15574 | 15573 |  |  |  |  |  |  |  |  |  |  |
|  | **15588** |  |  | **15589** |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  | 15633 |  |  |  |  |
|  |  |  |  |  |  | **15692** |  | **15691** | **15691** |  | **15693** |
|  |  |  |  |  |  |  |  |  |  |  | 15768 |
|  |  |  |  |  |  |  | 15909 |  |  |  |  |
|  |  |  |  |  |  | **15933** | **15931** | **15932** | **15933** | **15932** | **15932** |
| **16066** | **16066** | **16066** | **16065** | **16066** | **16064** | **16065** | **16068** | **16067** |  |  | **16019** |
|  |  |  |  | 16082 |  |  |  |  |  |  |  |
|  |  |  |  |  |  | 16203 |  |  |  |  |  |
|  |  |  | **16370** |  |  | **16370** | **16372** |  |  |  |  |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
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| | | | | 16405 | | | | | | | |
| **16428** | **16428** | **16427** | **16429** | **16426** | **16429** | **16428** | **16429** | **16429** | **16429** | **16430** | |
| | | | | | 16490 | | | | | | |
| | | | | 16517 | | | | | | | |
| | | | | 16567 | | | | | | | |
| | | | | | | 16600 | 16661 | | | | |
| | | | | | | **16711** | | | | **16710** | |
| | | 16896 | | | | | | | | | |
| | | | | | | | | 16904 | | 16903 | |
| | | | 16910 | 16910 | 16911 | | 16911 | 16911 | | | |
| | 16527 | | | | | | | | | | |
| **16937** | | | | | | | | | | | |
| | | | | | | **17024** | | | | | |
| | | | | | | | 17189 | | | | |
| 17307 | 17308 | | | | | | | | | | |
| | | | | | | | | | | | 17472 |
| | | | | | **17513** | **17514** | | **17517** | | | |
| **17529** | **17530** | | **17530** | **17530** | | | | | | | |
| | 17550 | | | | | | | | | | |
| | 17586 | | | | | | | | 17586 | | |
| | 17630 | | | | | | | | | | |
| | **17766** | | **17768** | **17766** | | **17768** | | | | | |
| | | | | | | | | | 17619 | | |
| | | | | | | | 17814 | | | | |
| | 17864 | | | | | | | | | | |
| **17904** | | | **17905** | **17905** | | | | | | | |
| | | | | | | | | | | **17958** | |
| | | | | | | **18161** | **18159** | **18162** | **18162** | **18162** | **18161** |
| | | | | | | | | | 18180 | | |
| | | | | 18207 | | | | | | | |
| | | | | | | | 18366 | | | | |
| | | | | | | | | 18510 | | 18511 | |
| | | | | | | | | | **18566** | **18564** | **18565** |
| | | | | | **18773** | **18773** | | | | **18774** | |
| | | 18866 | | | | | | | | | |
| | | | | | | | 18926 | | | | |
| | | | | **18932** | | **18933** | | | | | |
| | | | | | | | 18953 | | | | |
| | | | | | | | | | | | 19030 |
| | | | **19093** | | | | | | | | |
| | | | | | | 19381 | | | | | |
| | | | 19386 | | | | | | | | |
| | | | | | | 19487 | | | | | |
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| | | | 19722 | | | | | | | | |

| **2hr** | **3hr** | **4hr** | **6hr** | **8hr** | **12hr** | **16hr** | **20hr** | **24hr** | **36hr** | **48hr** | **72hr** |
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| **19878** | | | **19879** | **19878** | | **19878** | **19878** | **19878** | | | **19877** |
| | **20166** | | **20166** | **20166** | | **20165** | **20164** | | | **20165** | |
| | | | 20189 | | | | | | | | |
| | | | | | | | 20309 | | | | |
| | | | | | | 20379 | | | 20377 | 20377 | |
| | | | | | | | | | | 20475 | |
| | | | | | | 20685 | | | | | |
| | | 20698 | | | | | | | | | |
| | | | | | | | 20823 | | | | |
| | | | | | | | | | 20858 | | |
| | | | | 21221 | | | | | | | |
| | | | | 21289 | | | | | | | |
| | | | | | | | | | | | 21972 |
| | | | | | | | 22173 | | | | |
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| | | | | 22528 | | | | | | | |
| | | | | | | **22562** | | | | | |
| | | | | | | 22838 | | | | | |
| | | 23118 | | | | | | | | | |
| | 23236 | | | | | | | | | | |
| | 25078 | | | | | | | | | | |
| | | | 24347 | 24351 | | | | | | | |
| | | 24373 | | 24374 | | | | | | | |
| | | | | | | | | | 24804 | | |
| | | | | | | | | 25191 | | | |
| | | | 25370 | | | | | | | | |
| | | 25518 | | | | | | | | | |
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| | | | | | | | | | 27449 | | |
| | | | | | | | | | | | 27805 |
| | | | | | | | 28571 | | | | |
| | | | | | | | 29175 | | | | |
| | **29778** | **29776** | **29775** | **29777** | | | | | | | |
| | | 29873 | | | | | | | | | |
| | | 29945 | | | | | | | | | |
| | | | | | | | | | 30440 | | |
| | | | | | | 30464 | | | | | |
| | | 30604 | | | | | | | | | |
| | | | | 30705 | | | | | | | |
| **31091** | | **31094** | | | | | | | | | |
| | | | | 32809 | | | | | | | |
| | | | | | 33363 | | | | | | |
| | | | | | | | | | | | 33718 |
| | | | | | | | 35055 | | | | |
| | | | 35101 | | | | | | | | |

| 2hr | 3hr | 4hr | 6hr | 8hr | 12hr | 16hr | 20hr | 24hr | 36hr | 48hr | 72hr |
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|  |  |  |  |  | 35163 |  | 35159 |  |  |  |  |
| **35170** |  |  |  |  |  | **35175** |  |  | **35172** | **35171** | **35171** |
|  |  |  |  |  |  |  |  |  |  |  | 35196 |
|  |  |  |  |  |  |  |  |  |  | 35202 |  |
|  |  |  | 35325 |  |  |  |  | 35322 |  |  |  |
|  |  |  |  |  | 35702 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  | **35913** |
|  |  |  |  |  |  | 36320 |  |  |  |  |  |
|  |  |  |  |  |  | 37447 |  |  |  |  |  |
|  |  |  |  |  | 38290 |  |  |  |  |  |  |
|  |  |  | 41381 |  |  |  |  |  |  |  |  |
|  |  | 42481 |  |  |  |  |  |  |  |  |  |
|  |  | 45298 |  |  |  |  |  |  |  |  |  |
|  |  |  | 48218 |  |  |  |  |  |  |  |  |